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     (FILE 'HOME' ENTERED AT 15:03:43 ON 11 SEP 2003)

     FILE 'MEDLINE, EMBASE, BIOSIS, CAPLUS' ENTERED AT 15:03:57 ON 11 SEP 2003
L1       236767 S HEPARIN OR HEPARAN
L2       147971 S HERPES OR HSV
L3          856 S L1 AND L2
L4          430 DUP REM L3 (426 DUPLICATES REMOVED)

     FILE 'CAPLUS' ENTERED AT 15:05:47 ON 11 SEP 2003
L5        46369 S HEPARIN OR HEPARAN
L6        22923 S HERPES OR HSV
L7          206 S L5 AND L6
L8        37317 S GD
L9           25 S L7 AND L8
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L9 ANSWER 1 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2003:330667 CAPLUS
TITLE: A single amino acid substitution in the cytoplasmic tail of the glycoprotein B of herpes simplex virus 1 affects both syncytium formation and binding to intracellular heparan sulfate
AUTHOR(S): Diakidi-Kosta, A.; Michailidou, G.; Kontogounis, G.; Sivropoulou, A.; Arsenakis, M.
CORPORATE SOURCE: School of Biology, Development and Molecular Biology, Section of Genetics, Laboratory of General Microbiology, Aristotle University, Thessaloniki, 54124, Greece
SOURCE: Virus Research (2003), 93(1), 99-108
CODEN: VIREDF; ISSN: 0168-1702
PUBLISHER: Elsevier Science Ltd.
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Herpes simplex virus 1 (HSV-1) (S) is a spontaneous syncytial mutant derived from the prototype HSV-1(F) after extensive plaque purifn., and produces large syncytial plaques on Vero cells. Marker transfer expts. and DNA sequence anal. mapped the syncytial phenotype to a T-C base substitution at codon 787 of the cytoplasmic domain of mature gB, that results in Leu to Pro substitution and consequently belongs to the syn 3 locus. Both the cytoplasmic and the extracellular domains of gB are active in the fusion event since the addn. of anti-gB monoclonal antibodies that recognize the extracellular domain of gB prevent HSV-1(S) induced cell fusion. Similarly, gD also participates in cell fusion since addn. of anti-gD monoclonal antibodies also prevent HSV-1(S) induced cell fusion. Furthermore the glycoproteins B and D formed complexes in cells infected with mutant or wild type viruses. The amt. of gB bound to total heparan sulfate is lower in the mutant than in the wild type strain. This difference becomes particularly profound when gB is assocd. with a portion of heparan sulfate intercalated to the membranes. The discrepancy in the binding of the mutant and wild type gB to heparan sulfate may be related to the mechanism of cell fusion induced by HSV-1(S).
REFERENCE COUNT: 40 THERE ARE 40 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 2 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2002:720731 CAPLUS
DOCUMENT NUMBER: 138:34997
TITLE: Characterization of a Heparan Sulfate Octasaccharide That Binds to Herpes Simplex Virus Type 1 Glycoprotein D
AUTHOR(S): Liu, Jian; Shriver, Zach; Pope, R. Marshall; Thorp, Suzanne C.; Duncan, Michael B.; Copeland, Ronald J.; Raska, Christina S.; Yoshida, Keiichi; Eisenberg, Roselyn J.; Cohen, Gary; Linhardt, Robert J.; Sasisekharan, Ram
CORPORATE SOURCE: School of Pharmacy, Division of Medicinal Chemistry and Natural Products, University of North Carolina, Chapel Hill, NC, 27599, USA
SOURCE: Journal of Biological Chemistry (2002), 277(36), 33456-33467
CODEN: JBCHA3; ISSN: 0021-9258
PUBLISHER: American Society for Biochemistry and Molecular Biology
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Herpes simplex virus type 1 utilizes cell surface heparan sulfate as receptors to infect target cells. The unique heparan sulfate saccharide sequence offers the binding site for viral envelope proteins and plays crit. roles in assisting viral infections. A specific 3-O-sulfated heparan sulfate is known to facilitate the entry of herpes simplex virus 1 into cells. The 3-O-sulfated heparan sulfate is generated by the heparan sulfate D-glucosaminyl-3-O-sulfotransferase isoform 3 (3-OST-3), and it provides binding sites for viral glycoprotein D (gD). Here, we report the purifn. and structural characterization of an oligosaccharide that binds to gD. The isolated gD-binding site is an octasaccharide, and has a binding affinity to gD around 18 .mu.M, as detd. by affinity coelectrophoresis. The octasaccharide was prepd. and purified from a heparan sulfate oligosaccharide library that was modified by purified 3-OST-3 enzyme. The mol. mass of the isolated octasaccharide was detd. using both nanoelectrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry. The results from the sequence

anal. suggest that the structure of the octasaccharide is a heptasulfated octasaccharide. The proposed structure of the octasaccharide is .DELTA.UA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH23S6S. Given that the binding of 3-O-sulfated heparan sulfate to gD can mediate viral entry, our results provide structural information about heparan sulfate-assisted viral entry.

REFERENCE COUNT: 47 THERE ARE 47 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 3 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2002:357238 CAPLUS
DOCUMENT NUMBER: 137:18498
TITLE: Kinetic Analysis of Glycoprotein C of Herpes Simplex Virus Types 1 and 2 Binding to Heparin , Heparan Sulfate, and Complement Component C3b
AUTHOR(S): Rux, Ann H.; Lou, Huan; Lambris, John D.; Friedman, Harvey M.; Eisenberg, Roselyn J.; Cohen, Gary H.
CORPORATE SOURCE: Department of Microbiology, University of Pennsylvania, Philadelphia, PA, 19104, USA
SOURCE: Virology (2002), 294(2), 324-332
CODEN: VIRLAX; ISSN: 0042-6822
PUBLISHER: Elsevier Science
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Glycoprotein C (gC) from herpes simplex virus (HSV) facilitates virus entry by attaching the virion to host cell-surface heparan sulfate (HS). Although gC from HSV-1 (gC1) and from HSV-2 (gC2) bind to heparin, gC2 is believed to play a less significant role than gC1 in attachment of virus to cells. This attachment step is followed by the binding of gD to one of several cellular receptors. GC also plays an important role in immune evasion by binding to the C3b fragment of the host complement component C3. Yet, although both gC1 and gC2 protect HSV against complement-mediated neutralization, only gC on HSV-1-infected cells acts as a receptor for C3b. The authors used optical biosensor technol. to quantitate the affinities (KD) and the stabilities (koff) between both serotypes of gC with heparin, HS, and C3b to address 3 questions concerning gC interactions. First, can differences in affinity or stability account for differences between the contributions of HSV-1 and HSV-2 gC in attachment. The authors' data show that the gC2-HS complex is highly unstable (koff = 0.2 s-1) compared to the gC1-HS complex (koff = 0.003 s-1), suggesting why gC2 may not play an important role in attachment of virus to cells as does gC1. Second, does gC2 have a lower affinity for C3b than does gC1, thereby explaining the lack of C3b-receptor activity on HSV-2 infected cells. Surprisingly, gC2 had a 10-fold higher affinity for C3b compared to gC1, so this functional difference in serotypes cannot be accounted for by affinity. Third, do differences in gC-HS and gD-receptor affinities support a model of HSV entry in which the gC-HS interaction is of lower affinity than the gD-receptor interaction. The authors' biosensor results indicate that gC has a higher affinity for HS than gD does for cellular receptors HveA (HVEM) and HveC (nectin-1).

REFERENCE COUNT: 57 THERE ARE 57 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 4 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2001:775449 CAPLUS
DOCUMENT NUMBER: 136:33840
TITLE: Portable sulphotransferase domain determines sequence specificity of heparan sulphate 3-O-sulphotransferases
AUTHOR(S): Yabe, Tomio; Shukla, Deepak; Spear, Patricia G.; Rosenberg, Robert D.; Seeberger, Peter H.; Shworak, Nicholas W.
CORPORATE SOURCE: Angiogenesis Research Center, Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA, 02215, USA
SOURCE: Biochemical Journal (2001), 359(1), 235-241
CODEN: BIJOAK; ISSN: 0264-6021
PUBLISHER: Portland Press Ltd.
DOCUMENT TYPE: Journal
LANGUAGE: English
AB 3-O-Sulfates are the rarest substituent of heparan sulfate and are therefore ideally suited to the selective regulation of biol. activities. Individual isoforms of heparan sulfate D-glucosaminyl 3-O-sulfotransferase (3-OST) exhibit sequence-specific action, which creates heparan sulfate structures with distinct biol. functions. For example, 3-OST-1 preferentially generates binding

sites for anti-thrombin, whereas 3-OST-3 isoforms create binding sites for the gD envelope protein of **herpes** simplex virus 1 (**HSV**-1), which enables viral entry. 3-OST enzymes comprise a presumptive sulfotransferase domain and a divergent N-terminal region. To localize determinants of sequence specificity, we conducted domain swaps between cDNA species. The N-terminal region of 3-OST-1 was fused with the sulfotransferase domain of 3-OST-3A to generate N1-ST3A. Similarly, the N-terminal region of 3-OST-3A was fused to the sulfotransferase domain of 3-OST-1 to generate N3A-ST1. Wild-type and chimeric enzymes were transiently expressed in COS-7 cells and exts. were analyzed for selective generation of binding sites for anti-thrombin. 3-OST-1 was 270-fold more efficient at forming anti-thrombin-binding sites than 3-OST-3A, indicating its significantly greater selectivity for substrates that can be 3-O-sulfated to yield such sites. N3A-ST1 was as active as 3-OST-1, whereas the activity of N1-ST3A was as low as that of 3-OST-3A. Anal. of Chinese hamster ovary cell transfectants revealed that only 3-OST-3A and N1-ST3A generated gD-binding sites and conveyed susceptibility to infection by **HSV**-1. Thus sequence-specific properties of 3-OSTs are defined by a self-contained sulfotransferase domain and are not directly influenced by the divergent N-terminal region.

REFERENCE COUNT: 45 THERE ARE 45 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 5 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2001:561500 CAPLUS
DOCUMENT NUMBER: 135:271047
TITLE: **Herpes** simplex virus glycoprotein D bound to the human receptor HveA
AUTHOR(S): Carfi, Andrea; Willis, Sharon H.; Whitbeck, J. Charles; Krummenacher, Claude; Cohen, Gary H.; Eisenberg, Roselyn J.; Wiley, Don C.
CORPORATE SOURCE: Department of Medicine, Howard Hughes Medical Institute, Children's Hospital, Boston, MA, 02115, USA
SOURCE: Molecular Cell (2001), 8(1), 169-179
CODEN: MOCEFL; ISSN: 1097-2765
PUBLISHER: Cell Press
DOCUMENT TYPE: Journal
LANGUAGE: English

AB **Herpes** simplex virus (**HSV**) infection requires binding of the viral envelope glycoprotein D (gD) to cell surface receptors. We report the X-ray structures of a sol., truncated ectodomain of gD both alone and in complex with the ectodomain of its cellular receptor HveA. Two bound anions suggest possible binding sites for another gD receptor, a 3-O-sulfonated **heparan** sulfate. Unexpectedly, the structures reveal a V-like Ig fold at the core of gD that is closely related to cellular adhesion mols. and flanked by large N- and C-terminal extensions. The receptor binding segment of gD, an N-terminal hairpin, appears conformationally flexible, suggesting that a conformational change accompanying binding might be part of the viral entry mechanism.

REFERENCE COUNT: 64 THERE ARE 64 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 6 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2001:152481 CAPLUS
DOCUMENT NUMBER: 134:212717
TITLE: Pharmaceutical preparations for the inhibition of **herpes** simplex virus 1 entry
INVENTOR(S): Shukla, Deepak; Liu, Jian; Rosenberg, Robert D.; Spear, Patricia G.
PATENT ASSIGNEE(S): Massachusetts Institute of Technology, USA; Northwestern University
SOURCE: PCT Int. Appl., 29 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2001013910	A2	20010301	WO 2000-US23288	20000825
WO 2001013910	A3	20011101		

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY,

DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG
PRIORITY APPLN. INFO.: US 1999-150743P P 19990825
US 2000-175347P P 20000110
AB Disclosed herein are polysaccharide prepns. enriched in 3-OST-3 modified **heparan** sulfate. Also disclosed are methods of treating **herpes** simplex viral type-1 infection using the pharmaceutical prepns. of the invention.
REFERENCE COUNT: 50 THERE ARE 50 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 7 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2001:16328 CAPLUS
DOCUMENT NUMBER: 134:219618
TITLE: Cell fusion induced by **herpes** simplex virus glycoproteins gB, **gD**, and gH-gL requires a **gD** receptor but not necessarily **heparan** sulfate
AUTHOR(S): Pertel, Peter E.; Fridberg, Alina; Parish, Mary L.; Spear, Patricia G.
CORPORATE SOURCE: Department of Medicine, Division of Infectious Diseases, Northwestern University Medical School, Chicago, IL, 60611, USA
SOURCE: Virology (2001), 279(1), 313-324
CODEN: VIRLAX; ISSN: 0042-6822
PUBLISHER: Academic Press
DOCUMENT TYPE: Journal
LANGUAGE: English
AB To characterize cellular factors required for **herpes** simplex virus type 1 (**HSV**-1)-induced cell fusion, we used an efficient and quant. assay relying on expression of **HSV**-1 glycoproteins in transfected cells. We showed the following: (1) Cell fusion depended not only on expression of four viral glycoproteins (gB, **gD**, and gH-gL), as previously shown, but also on expression of cell surface entry receptors specific for **gD**. (2) Cell fusion required expression of all four glycoproteins in the same cell. (3) **Heparan** sulfate was not required for cell fusion. (4) Coexpression of receptor with the four glycoproteins in the same cell reduced fusion activity, indicating that interaction of **gD** and receptor can limit polykaryocyte formation. Overall, the viral and cellular determinants of **HSV** -1-induced cell fusion are similar to those for viral entry, except that **HSV**-1 entry is significantly enhanced by binding of virus to cell surface **heparan** sulfate. (c) 2001 Academic Press.
REFERENCE COUNT: 71 THERE ARE 71 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 8 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 2000:744828 CAPLUS
DOCUMENT NUMBER: 134:309317
TITLE: The novel receptors that mediate the entry of **herpes** simplex viruses and animal alphaherpesviruses into cells
AUTHOR(S): Campadelli-Fiume, Gabriella; Cocchi, Francesca; Menotti, Laura; Lopez, Marc
CORPORATE SOURCE: Department of Experimental Pathology, Section on Microbiology and Virology, University of Bologna, Bologna, 40126, Italy
SOURCE: Reviews in Medical Virology (2000), 10(5), 305-319
CODEN: RMVIEW; ISSN: 1052-9276
PUBLISHER: John Wiley & Sons Ltd.
DOCUMENT TYPE: Journal; General Review
LANGUAGE: English
AB A review with 80 refs. An extended array of cell surface mols. serve as receptors for **HSV** entry into cells. In addn. to the **heparan** sulfate glycosaminoglycans, which mediate the attachment of virion to cells, **HSV** requires an entry receptor. The repertoire of entry receptors into human cells includes mols. from three structurally unrelated mol. families. They are (i) HveA (herpesvirus entry mediator A), (ii) members of the nectin family, (iii) 3-O-sulfated **heparan** sulfate. The mols. have different attributes and play potentially different roles in **HSV** infection and spread to human tissues. All the human entry receptors interact phys. with the virion envelope glycoprotein D (**gD**). (i) HveA is a member of the TNF-receptor family. It mediates entry of a restricted range of **HSV** strains. Its expression is restricted to few lineages (e.g. T-lymphocytes). (ii) The human nectin1.alpha. (HIgR), nectin1.delta. (PRR1-HveC), and the nectin2.alpha. (PRR2.alpha.-HveB) and nectin2.delta. (PRR2.delta.) belong to the Ig superfamily. They are homologs of the poliovirus receptor (CD155), with which they share the overall structure of the ectodomain. The human nectin1.alpha.-.delta. are broadly expressed

in cell lines of different lineages, are expressed in human tissue targets of HSV infection, serve as receptors for all HSV-1 and HSV-2 strains tested and mediate entry not only of free virions, but also cell-to-cell spread of virus. (iii) The 3-O-sulfated heparan sulfate is expressed in some selected human cell lines (e.g. endothelial and mast cells) and human tissues, and mediates entry of HSV-1, but not HSV-2. The human nectin2.alpha. and nectin2.delta. serve as receptors for a narrow range of viruses. A characteristic of the human nectin1.alpha.-.delta. is the promiscuous species non-specific receptor activity towards the animal alphaherpesviruses, pseudorabies virus (PrV) and bovine herpesvirus 1 (BHV-1). By contrast with the human nectin1.delta., its murine homolog (mNectin1.delta.) does not bind gD at detectable level, yet it mediates entry of HSV, as well as of PrV and BHV-1. This provides the first example of a mediator of HSV entry independent of a detectable interaction with gD.

REFERENCE COUNT: 80 THERE ARE 80 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 9 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1999:673913 CAPLUS
DOCUMENT NUMBER: 132:21694
TITLE: A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry
AUTHOR(S): Shukla, Deepak; Liu, Jian; Blaiklock, Peter; Shworak, Nicholas W.; Bai, Xiaomei; Esko, Jeffrey D.; Cohen, Gary H.; Eisenberg, Roselyn J.; Rosenberg, Robert D.; Spear, Patricia G.
CORPORATE SOURCE: Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, IL, 60611, USA
SOURCE: Cell (Cambridge, Massachusetts) (1999), 99(1), 13-22
CODEN: CELLB5; ISSN: 0092-8674
PUBLISHER: Cell Press
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Herpes simplex virus type 1 (HSV-1) binds to cells through interactions of viral glycoproteins gB and gC with heparan sulfate chains on cell surface proteoglycans. This binding is not sufficient for viral entry, which requires fusion between the viral envelope and cell membrane. Here, the authors show that heparan sulfate modified by a subset of the multiple D-glucosaminyl 3-O-sulfotransferase isoforms provides sites for the binding of a third viral glycoprotein, gD, and for initiation of HSV-1 entry. The authors conclude that susceptibility of cells to HSV -1 entry depends on (1) presence of heparan sulfate chains to which virus can bind and (2) 3-O-sulfation of specific glucosamine residues in heparan sulfate to generate gD-binding sites or the expression of other previously identified gD -binding receptors.
REFERENCE COUNT: 48 THERE ARE 48 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 10 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1999:344861 CAPLUS
DOCUMENT NUMBER: 131:4240
TITLE: Immunoglobulin molecules having a synthetic variable region and modified specificity
INVENTOR(S): Burch, Ronald M.
PATENT ASSIGNEE(S): Euro-Celtique, S.A., Bermuda
SOURCE: PCT Int. Appl., 123 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9925378	A1	19990527	WO 1998-US24302	19981113
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
CA 2309990	AA	19990527	CA 1998-2309990	19981113
CA 2310269	AA	19990527	CA 1998-2310269	19981113
WO 9925379	A1	19990527	WO 1998-US24303	19981113

W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

AU 9914597	A1	19990607	AU 1999-14597	19981113
AU 763029	B2	20030710		
AU 9914598	A1	19990607	AU 1999-14598	19981113
AU 737457	B2	20010823		
EP 1030684	A1	20000830	EP 1998-958584	19981113

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI

EP 1032420	A1	20000906	EP 1998-958583	19981113

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI

JP 2001526021	T2	20011218	JP 2000-520811	19981113
BR 9815289	A	20011226	BR 1998-15289	19981113
BR 9815580	A	20020129	BR 1998-15580	19981113
JP 2002507544	T2	20020312	JP 2000-520812	19981113
ZA 9900048	A	19990708	ZA 1999-48	19990105
ZA 9900049	A	20000309	ZA 1999-49	19990105
US 2002028469	A1	20020307	US 2001-963232	20010926
WO 2003026879	A2	20030403	WO 2002-US27446	20020828
WO 2003026879	A3	20030605		

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

PRIORITY APPLN. INFO.:	US 1997-65716P	P 19971114
	US 1998-81403P	P 19980410
	US 1998-191780	A1 19981113
	WO 1998-US24302	W 19981113
	WO 1998-US24303	W 19981113
	US 2001-963232	A 20010926

AB The invention provides modified Ig mols., particularly antibodies, that immunospecifically bind a first member of a binding pair which binding pair consists of the first member and a second member, which Igs have a variable domain contg. one or more complimentary detg. regions that contain the amino acid sequence of a binding site for the second member of the binding pair. The first member is a tumor antigen or an antigen of an infectious disease agent, and the second member is a mol. on the surface of an immune cell. The invention further provides for therapeutic and diagnostic use of the modified Ig.

REFERENCE COUNT: 13 THERE ARE 13 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 11 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN

ACCESSION NUMBER:	1999:217578 CAPLUS
DOCUMENT NUMBER:	131:72062
TITLE:	**Herpes** Simplex Virus Entry Is Associated with Tyrosine Phosphorylation of Cellular Proteins
AUTHOR(S):	Qie, Lixin; Marcellino, Daniel; Herold, Betsy C.
CORPORATE SOURCE:	Department of Pediatric Infectious Diseases, Mount Sinai Medical School, New York, NY, 10029-6574, USA
SOURCE:	Virology (1999), 256(2), 220-227 CODEN: VIRLAX; ISSN: 0042-6822
PUBLISHER:	Academic Press
DOCUMENT TYPE:	Journal
LANGUAGE:	English

AB The initial step in **herpes** simplex virus (**HSV**) entry is binding of virion glycoprotein (g)C and/or gB to cell surface **heparan** sulfate. After this initial attachment, **gD** interacts with cell surface receptor or receptors, and the virion envelope fuses with the cell membrane. Fusion requires viral glycoproteins gB, gD, gL, and gH, but the cellular factors that participate in or the pathways activated by viral entry have not been defined. To det. whether signal transduction pathways are triggered by viral-cell fusion, the authors examd. the assocn. of viral entry with tyrosine phosphorylation of cellular proteins. Using immunopptn. and Western blotting, the authors found that at least three cytoplasmic host cell proteins, designated p80, p104, and p140, become tyrosine phosphorylated

within 5-10 min after exposure to HSV-1 or HSV-2. However, no phosphorylation is detected when cells are exposed to a mutant virus deleted in gL that binds but fails to penetrate. Phosphorylation is restored when the gL-deletion virus is grown on a complementing cell line. Viral entry and the phosphorylation of p80, p104, and p140 are inhibited when cells are infected with virus in the presence of protein tyrosine kinase inhibitors. Taken together, these studies suggest that tyrosine phosphorylation of host cellular proteins is triggered by viral entry. (c) 1999 Academic Press.

REFERENCE COUNT: 41 THERE ARE 41 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 12 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1998:752060 CAPLUS
DOCUMENT NUMBER: 130:235693
TITLE: Stable attachment for herpes simplex virus penetration into human cells requires glycoprotein D in the virion and cell receptors that are missing for entry-defective porcine cells
AUTHOR(S): Perez, Aleida; Fuller, A. Oveta
CORPORATE SOURCE: 6736 Medical Sciences II, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI, 48109-0620, USA
SOURCE: Virus Research (1998), 58(1-2), 21-34
CODEN: VIREDF; ISSN: 0168-1702
PUBLISHER: Elsevier Science B.V.
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Clonal porcine kidney cell lines that are non-permissive for herpes simplex virus (HSV) infection produced five orders of magnitude less virus than human cells, contained heparin sulfate (HS), and are restricted only at HSV entry. By fluorescent activated cell sorting, we examd. HSV attachments to porcine and human cells. Stable attachment to susceptible human embryonic lung (HEL) cells occurred with infectious wild-type virus, complemented gD or gH mutant viruses, or non-infectious virus lacking gH. On HEL cells, mutant virus lacking gD bound to heparan sulfate, but failed to stably bind. None of these viruses stably attached to SK6-A7 cells, one of the non-permissive porcine cell clones. However, HSV could replicate in these cells when entry was mediated by polyethylene glycol. These results confirm that, in neutral pH entry of HSV, (i) multiple attachments to HS and non-HS components lead to penetration, (2) stable attachment before penetration is one required function of gD, but not gH, and (3) for stable attachment, gD interacts directly, or indirectly through another viral or cellular component, with receptors that are present on human cells, but absent for entry-defective porcine cells. Easily propagated clonal porcine cells are a novel resource to investigate stable attachment, the mol. mechanisms of gD functions, and the viral and cellular components that allow HSV entry and spread.

REFERENCE COUNT: 61 THERE ARE 61 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 13 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1998:484956 CAPLUS
DOCUMENT NUMBER: 129:133369
TITLE: Microporation of tissue for delivery of bioactive agents
INVENTOR(S): Eppstein, Jonathan A.
PATENT ASSIGNEE(S): Altea Technologies, Inc., USA; Eppstein, Jonathan A.
SOURCE: PCT Int. Appl., 168 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9829134	A2	19980709	WO 1997-US24127	19971230
WO 9829134	A3	19981015		
W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
EP 921840	A1	19990616	EP 1997-936041	19970703

EP 921840	B1	20030528			
R: AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE					
JP 2000513971	T2	20001024	JP 1998-504488	19970703	
AT 241405	E	20030615	AT 1997-936041	19970703	
AU 9856232	A1	19980731	AU 1998-56232	19971230	
EP 952850	A1	19991103	EP 1997-952676	19971230	
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI					
JP 2001512329	T2	20010821	JP 1998-530298	19971230	
PRIORITY APPLN. INFO.:			US 1996-778415	A2 19961231	
			WO 1997-US11670	A 19970703	
			US 1996-21212P	P 19960703	
			WO 1997-US24127	W 19971230	

AB A method of enhancing the permeability of a biol. membrane, including the skin or mucosa of an animal or the outer layer of a plant, to a permeant is described which utilizes microporation of selected depth and optionally .gtoreq.1 of sonic, electromagnetic, mech., and thermal energy and a chem. enhancer. Microporation is accomplished to form a micropore of selected depth in the biol. membrane and the porated site is contacted with the permeant. Addnl. permeation enhancement measures may be applied to the site to enhance the flux rate of a permeant, e.g. a drug, into an organism through the micropores and into targeted tissues within the organism; the parameters of these measures can be tailored to act selectively on specific tissue barriers. Microporation can also be used for minimally invasive or noninvasive monitoring of analytes in body fluids by enhancing their outward diffusion to the skin surface. Micropores .ltoreq.1000 .mu.m in diam. are produced by ablating the membrane with a heat source, a microlancet, a beam of sonic energy, a high-pressure jet of fluid, a short pulse of electricity, or a short light pulse emitted e.g. by a laser diode and focused on a site treated with a light-absorbing substance to generate heat at the site. The energy source is modulated to minimize sensory perception of the process, e.g. by use of energy pulses alternated with cooling or recovery periods. Pore depth is detd. by measuring the impedance properties of the tissue. Thus, a small drop of Cu phthalocyanine suspension in iso-PrOH was evapd. on transparent adhesive tape which was then attached to the skin of a volunteer and irradiated with pulsed laser light to produce a pore in the stratum corneum extending to the epidermis. Interstitial fluid (5 .mu.L) collected from the pore was analyzed for glucose with a glucometer in normal and diabetic subjects. The av. temporal lag between blood and interstitial fluid glucose levels in response to a glucose load was only 6.2 min; an equation relating blood and interstitial fluid glucose levels is presented. In another expt., a soln. contg. lidocaine and a permeation enhancer was applied to a grid of similarly produced micropores in the skin to produce numbness; permeation was further increased by application of ultrasound through a transducer.

L9 ANSWER 14 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN

ACCESSION NUMBER:	1998:482355 CAPLUS
DOCUMENT NUMBER:	129:242243
TITLE:	The role of **herpes** simplex virus glycoproteins in the virus replication cycle
AUTHOR(S):	Rajcani, J.; Vojvodova, A.
CORPORATE SOURCE:	Institute of Virology, Slovak Academy of Sciences, Bratislava, 842 46, Slovakia
SOURCE:	Acta Virologica (English Edition) (1998), 42(2), 103-118 CODEN: AVIRA2; ISSN: 0001-723X
PUBLISHER:	Slovak Academic Press Ltd.
DOCUMENT TYPE:	Journal; General Review
LANGUAGE:	English

AB A review with 95 refs. At least nine of the eleven **herpes** simplex virus (**HSV**) glycoproteins so far known have been widely characterized as regards their role in the virus replication cycle. During early virus-to-cell adsorption ("adsorption"), glycoprotein C (gC) interacts with the glycosaminoglycan (GAG) **heparan** sulfate (HS), located on the cell membrane surface. This interaction is labile until other glycoproteins such as B and D (gB and **gD**) begin to participate in the entry process. GB also harbors a site for interaction with GAGs, while **gD** provides a stabile attachment to cellular receptors ("receptors") such as the herpesvirus entry mediator (HVEM). Late adsorption is assocd. with a conformation change of **gD** occurring after the receptor binding, a step followed by interaction of **gD** with the gH/gL heterodimer (complex). Fusion domains of the gH/gL complex and gB enable the pH-independent virus-into-cell penetration ("penetration"). The gE/gI complex and gM interact with the receptors at cell junctions to facilitate cell-to-cell spread of the virus along the basolateral surface of polarized cells and/or a similar intercellular spread in nonpolarized cells by avoiding virion release. GK, the only so

far known HSV-coded glycoprotein which is not incorporated into virions, plays an essential role in the virus capsid envelopment at the nuclear membrane and in the virion transport to the cell surface. Unusually large polykaryocytes arise due to mutations in syn (syncytium) loci of the viral genome, which were mapped to UL53 (syn1) and UL27 (syn3) genes coding for gK and gB, resp., while the genes UL20 and UL24 (both syn5) code for nonglycosylated cell membrane-assocd. proteins ("membrane proteins"). The products of nonmutated syn genes either downregulate the fusion of plasma membranes of infected cells ("membrane fusion") or protect them from undesirable fusion events.

REFERENCE COUNT: 195 THERE ARE 195 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L9 ANSWER 15 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1997:627572 CAPLUS
DOCUMENT NUMBER: 127:305103
TITLE: Partial resistance to gD-mediated interference conferred by mutations affecting herpes simplex virus type 1 gC and gK
AUTHOR(S): Pertel, Peter E.; Spear, Patricia G.
CORPORATE SOURCE: Division of Infectious Disease, Department of Medicine, Northwestern University Medical School, Chicago, IL, 60611, USA
SOURCE: Journal of Virology (1997), 71(10), 8024-8028
CODEN: JOVIAM; ISSN: 0022-538X
PUBLISHER: American Society for Microbiology
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Cells expressing herpes simplex virus (HSV) gD can be resistant to HSV entry as a result of gD -mediated interference. HSV strains differ in sensitivity to this interference, which blocks viral penetration but not binding. Previous studies have shown that mutations or variations in virion-assocd. gD can confer resistance to gD-mediated interference. Here, it is shown that HSV-1 mutants selected for enhanced ability to bind and penetrate in the presence of inhibitory concns. of heparin were partially resistant to gD-mediated interference. The resistance was largely due to the presence of two mutations: one in gC (the major heparin-binding glycoprotein) resulting in the absence of gC expression and the other in gK resulting in a syncytial phenotype. The results imply that heparin selected for mutants with altered postbinding requirements for entry. Resistance to gD-mediated interference conferred by mutations affecting gC and gK has not been previously described.

L9 ANSWER 16 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1997:56489 CAPLUS
DOCUMENT NUMBER: 126:101546
TITLE: Specificity and affinity of binding of herpes simplex virus type 2 glycoprotein B to glycosaminoglycans
AUTHOR(S): Williams, Richard K.; Straus, Stephen E.
CORPORATE SOURCE: Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, MD, 20892, USA
SOURCE: Journal of Virology (1997), 71(2), 1375-1380
CODEN: JOVIAM; ISSN: 0022-538X
PUBLISHER: American Society for Microbiology
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Herpes simplex virus type 2 (HSV-2) interacts with cell surface glycosaminoglycans during virus attachment. Glycoprotein B of HSV-2 can potentially mediate the interaction between the virion and cell surface glycosaminoglycans. To det. the specificity, kinetics, and affinity of these interactions, we used plasmon resonance-based biosensor technol. to measure HSV-2 glycoprotein binding to glycosaminoglycans in real time. The recombinant sol. ectodomain of HSV-2 gB (gB2) but not the sol. ectodomain of HSV-2 gD bound readily to biosensor surfaces coated with heparin. The affinity consts. (Kds) were detd. for gB2 (Kd = 7.7 .times. 10-7 M) and for gB2.DELTA.TM (Kd = 9.9 .times. 10-7 M), a recombinant sol. form of HSV-2 gB in which only its transmembrane domains has been deleted. GB2 binding to the heparin surface was competitively inhibited by low concns. of heparin (50% ED = 0.08 .mu.g/mL). Heparan sulfate and dermatan sulfate glycosaminoglycans have each been suggested as cell surface receptors for HSV. Our biosensor analyses showed that both heparan sulfate and dermatan sulfate inhibited gB2 binding (ED50 = 1 to 5 .mu.g/mL), indicating that gB2 interacts with both

heparin-like and dermatan sulfate glycosaminoglycans. Chondroitin sulfate A, in contrast, inhibited gB2 binding to heparin only at high levels (ED50 = 65 .mu.g/mL). The affinity and specificity of gB2 binding to glycosaminoglycans demonstrated in these studies support its role in the initial binding of HSV-2 to cells bearing heparan sulfate or dermatan sulfate glycosaminoglycans.

L9 ANSWER 17 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1997:31734 CAPLUS
DOCUMENT NUMBER: 126:72496
TITLE: Influence of glycoproteins B, C and D on the conversion of virus-to-cell attachment from heparin sensitivity to resistance
AUTHOR(S): Seck, T.; Koch, O.; Lingen, M.; Falke, D.
CORPORATE SOURCE: Institute Virology, Johannes Gutenberg University, Mainz, D-55101, Germany
SOURCE: Acta Virologica (English Edition) (1996), 40(4), 179-185
CODEN: AVIRA2; ISSN: 0001-723X
PUBLISHER: Slovak Academic Press
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Glycoprotein C-neg. (gC-) mutants of herpes simplex virus type 1 (HSV-1) derived from strains KOS and ANGpath were used to analyze the influence of sol. heparin on the phase of adsorption/attachment of HSV-1 to cells. A dose of 200 .mu.g/mL heparin given 20 min after infection of cells with the gC-pos. (gC+) strains KOS and ANGpath at 4.degree.C reduced the adsorption of infective particles to 20 - 30% of the controls. A weaker heparin effect was obsd. with gC- mutants. However, the gC- mutants also exhibited a short heparin-sensitive phase. Mutations in amino acids of gB or gD at positions 854 or 25 and 27, resp., did not alter the attachment capacities of these HSV mutants in the presence of heparin despite their peculiar fusion properties and resistance to sol. gD. We conclude that HSV-1 strains exhibit a heparin-resistant phase of attachment, which is detd. by gC. Lack of gC delays the heparin-resistant attachment phase of HSV-1 to cells.

L9 ANSWER 18 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1995:691304 CAPLUS
DOCUMENT NUMBER: 123:193473
TITLE: Characterization of cell-binding properties of bovine herpesvirus 1 glycoproteins B, C, and D: identification of a dual cell-binding function of gB
AUTHOR(S): Li, Yuanhao; van Drunen Little-van den Hurk, Sylvia; Babiuk, Lorne A.; Liang, Xiaoping
CORPORATE SOURCE: Dep. Veterinary Microbiol., Univ. Saskatchewan, Saskatoon, SK, S7N 0W0, Can.
SOURCE: Journal of Virology (1995), 69(8), 4758-68
CODEN: JOVIAM; ISSN: 0022-538X
PUBLISHER: American Society for Microbiology
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Previous studies have suggested that the attachment of bovine herpesvirus 1 (BHV-1) to permissive cells is mediated by its major glycoproteins B (gB), C (gC), and D (gD). In order to gain further insight into the mechanism of the BHV-1 attachment process, authentic gB, gC, and gD from BHV-1-infected cells and membrane anchor-truncated sol. gB, gC, and gD from stably transfected cell lines were purified by affinity chromatog. and their cell-binding properties were examd. on Madin-Darby bovine kidney cells. All of the glycoproteins tested exhibited saturable binding to Madin-Darby bovine kidney cells. Addn. of exogenous heparin or treatment of cells with heparinase to remove cellular heparan sulfate (HS) prevented both gC and gB from binding to cells but had no effect on gD binding. An assessment of competition between gB, gC, and gD for cell binding revealed that gC inhibited gB binding, whereas other combinations showed no effect. Cell-bound gC could be dissocd. by heparin or heparinase treatment. The response of bound gB to heparin and heparinase treatments differed for the authentic and sol. forms; while sol. gB was susceptible to the treatment, a significant portion of cell-bound authentic gB was resistant to the treatment. Binding affinity anal. showed that sol. gB and both forms of gC and gD each had single binding kinetics with comparable dissocn. consts. (Kds) ranging from 1.5 .times. 10-7 to 5.1 .times. 10-7 M, whereas authentic gB exhibited dual binding kinetics with Kd1 = 5.2 .times. 10-7 M and Kd2 = 4.1 .times. 10-9 M. These results demonstrate that BHV-1 gC binds only to cellular HS, gD binds to a non-HS component, and gB initially binds to HS and then binds with high affinity to a non-HS receptor. While

authentic gB inhibited viral plaque formation, sol. gB, which retains the HS-binding property but lacks the high-affinity binding property, was defective in this respect. These results suggest that the interaction between gB and its high-affinity receptor may play a crit. role in the virus entry process.

L9 ANSWER 19 OF 25. CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1995:622317 CAPLUS
DOCUMENT NUMBER: 123:80699
TITLE: Interaction of **herpes** simplex virus glycoprotein gC with mammalian cell surface molecules
AUTHOR(S): Tal-Singer, Ruth; Peng, Charline; Ponce de Leon, Manuel; Abrams, William R.; Banfield, Bruce W.; Tufaro, Frank; Cohen, Gary H.; Eisenberg, Roselyn J.
CORPORATE SOURCE: Dep. Microbiology, Univ. Pennsylvania, Philadelphia, PA, USA
SOURCE: Journal of Virology (1995), 69(7), 4471-83
CODEN: JOVIAM; ISSN: 0022-538X
PUBLISHER: American Society for Microbiology
DOCUMENT TYPE: Journal
LANGUAGE: English
AB The entry of **herpes** simplex virus (**HSV**) into mammalian cells is a multistep process beginning with an attachment step involving glycoproteins gC and gB. A second step requires the interaction of glycoprotein **gD** with a cell surface mol. We explored the interaction between gC and the cell surface by using purified proteins in the absence of detergent. Truncated forms of gC and **gD**, gC1(457t), gC2(426t), and gD1(306t), lacking the transmembrane and carboxyl regions were expressed in the baculovirus system. We studied the ability of these proteins to bind to mammalian cells, to bind to immobilized **heparin**, to block **HSV** type 1 (**HSV** -1) attachment to cells, and to inhibit plaque formation by **HSV** -1. Each of these gC proteins bound to conformation-dependent monoclonal antibodies and to human complement component C3b, indicating that they maintained the same conformation of gC proteins expressed in mammalian cells. Biotinylated gC1(457t) and gC2(426t) each bind to several cell lines. Binding was inhibited by an excess of unlabeled gC but not by **gD**, indicating specificity. The attachment of gC to cells involves primarily **heparan** sulfate proteoglycans, since heparitinase treatment of cells reduced gC binding by 50% but had no effect on **gD** binding. Moreover, binding of gC to two **heparan** sulfate-deficient L-cell lines, gro2C and sog9, both of which are mostly resistant to **HSV** infection, was markedly reduced. Purified gD1(306t), however, bound equally well to the two mutant cell lines. In contrast, satg. amts. of gC1(457t) interfered with **HSV**-1 attachment to cells but failed to block plaque formation, suggesting a role for gC in attachment but not penetration. A mutant form of gC lacking residues 33 to 123, gC1(.DELTA.33-123t), expressed in the baculovirus system, bound significantly less well to cells than did gC1(457t) and competed poorly with biotinylated gC1(457t) for binding. These results suggest that residues 33 to 123 are important for gC attachment to cells. In contrast, both the mutant and wild-type forms of gC bound to immobilized **heparin**, indicating that binding of these proteins to the cell surface involves more than a simple interaction with **heparin**. To det. that the contribution of the N-terminal region of gC is important for **HSV** attachment, we compared several properties of a mutant **HSV**-1 which contains gC lacking amino acids 33 to 123 to those of its parental virus, which contains full-length gC. The mutant bound less well to cells than the parental virus but exhibited normal growth properties. While we cannot rule out the possibility that other regions of gC contribute to its function in attachment, our studies show that the N terminus of gC is important for efficient attachment to cells.

L9 ANSWER 20 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1995:568135 CAPLUS
DOCUMENT NUMBER: 122:310180
TITLE: Sequential isolation of proteoglycan synthesis mutants by using **herpes** simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway
AUTHOR(S): Banfield, Bruce W.; Leduc, Yves; Esford, Lesley; Schubert, Kathryn; Tufaro, Frank
CORPORATE SOURCE: Dep. Microbiol. Immunology, Univ. British Columbia, Vancouver, V6T 1Z3, Can.
SOURCE: Journal of Virology (1995), 69(6), 3290-8
CODEN: JOVIAM; ISSN: 0022-538X
PUBLISHER: American Society for Microbiology
DOCUMENT TYPE: Journal
LANGUAGE: English

AB A novel mouse L-cell mutant cell line defective in the biosynthesis of glycosaminoglycans was isolated by selection for cells resistant to herpes simplex virus (HSV) infection. These cells, termed sog9, were derived from mutant parental gro2C cells, which are themselves defective in heparan sulfate biosynthesis and 90% resistant to HSV type 1 (HSV-1) infection compared with control L cells (S. Gruenheid et al., 1993). Here the authors show that sog9 cells exhibit a 3-order-of-magnitude redn. in susceptibility to HSV-1 compared with control L cells. In steady-state labeling expts., sog9 cells accumulated almost no [35S]sulfate-labeled or [6-3H]glucosamine-labeled glycosaminoglycans, suggesting that the initiation of glycosaminoglycan assembly was specifically reduced in these cells. Despite these defects, sog9 cells were fully susceptible to vesicular stomatitis virus (VSV) and permissive for both VSV and HSV replication, assembly, and egress. HSV plaques formed in the sog9 monolayers in proportion to the amt. of input virus, suggesting the block to infection was in the virus entry pathway. More importantly, HSV-1 infection of sog9 cells was not significantly reduced by sol. heparan sulfate, indicating that infection was glycosaminoglycan independent. Infection was inhibited by sol. gD -1, however, which suggests that glycoprotein gD plays a role in the infection of this cell line. The block to sog9 cell infection by HSV-1 could be eliminated by adding sol. dextran sulfate to the inoculum, which may act by stabilizing the virus at the sog9 cell surface. Thus, sog9 cells provide direct genetic evidence for a proteoglycan-independent entry pathway for HSV-1, and results with these cells suggest that HSV-1 is a useful reagent for the direct selection of novel animal cell mutants defective in the synthesis of cell surface proteoglycans.

L9 ANSWER 21 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1993:667835 CAPLUS
DOCUMENT NUMBER: 119:267835
TITLE: Herpes simplex virus type 1 and pseudorabies virus bind to a common saturable receptor on Vero cells that is not heparan sulfate
AUTHOR(S): Lee, Wen Chi; Fuller, A. Oveta
CORPORATE SOURCE: Med. Sch., Univ. Michigan, Ann Arbor, MI, 48109-0620, USA
SOURCE; Journal of Virology (1993), 67(9), 5088-97
CODEN: JOVIAM; ISSN: 0022-538X
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV) infect different natural hosts but are very similar in structure, replicative cycle, and entry into cultured cells. The authors detd. whether HSV-1 and PRV use the same cellular components during entry into Vero cells, which are highly susceptible to each virus but are not from native hosts for either. UV-inactivated virions of either HSV-1 or PRV could sat. cell surfaces to block infection of challenge HSV-1 or PRV. In the presence of satg. levels for infection of either virus, radiolabeled virus bound well and in a heparin-sensitive manner. This result shows that heparan sulfate proteoglycans on Vero cells are not the limiting cellular component. To identify the virus component required for blocking, the authors used an HSV-1 null mutant virus lacking gB, gD , or gH as blocking virus. Virions lacking gB were able to block infection of challenge virus to the same level as did virus contg. gB. In contrast, virions lacking gD lost all and most of the ability to block infection of HSV-1 and PRV, resp. HSV-1 lacking gH and PRV lacking gp50 also were less competent in blocking infection of challenge virus. Thus, HSV-1 and PRV bind to a common receptor for infection of Vero cells. Although both viruses bind a heparin -like cell component on many cells, including Vero cells, they also attach to a different and limited cell surface component that is bound at least by HSV-1 gD and possibly gH and to some degree by PRV gp50 but not gB. These results clearly demonstrate binding of both HSV-1 and PRV to a common cell receptor that is not heparan sulfate and demonstrate that several types of attachment occur for both viruses during infectious entry.

L9 ANSWER 22 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1993:189026 CAPLUS
DOCUMENT NUMBER: 118:189026
TITLE: Herpes simplex virus type 1-induced hemagglutination: Glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate
AUTHOR(S): Trybala, Edward; Svennerholm, Bo; Bergstroem, Tomas; Olofsson, Sigvard; Jeansson, Stig; Goodman, Jesse L.

CORPORATE SOURCE: Sch. Med., Univ. Minnesota, Minneapolis, MN, 55451, USA
SOURCE: Journal of Virology (1993), 67(3), 1278-85
CODEN: JOVIAM; ISSN: 0022-538X
DOCUMENT TYPE: Journal
LANGUAGE: English

AB The authors recently reported that **herpes** simplex virus type 1 (**HSV**-1) can cause agglutination of murine erythrocytes (E. Trybala, et al, 1990). The present study demonstrates moieties at the surface of erythrocytes. Hemagglutination was found to be a common property of all gC-expressing lab. strains and clin. isolates of **HSV**-1 tested. Mutants of **HSV**-1 deficient in glycoprotein C caused no specific hemagglutination, whereas their derivs. transfected with a functional gC-1 gene, thus reconstituting gC expression, regained full hemagglutinating activity. Hemagglutination activity was inhibited by antibodies against gC-1 but not by antibodies with specificity for glycoproteins gB, gD, or gE or by murine antiserum raised against the MP strain of **HSV**-1, which is gC deficient. Finally, purified gC-1 protein, like whole **HSV**-1 virions, showed high hemagglutinating activity which was inhibited by **heparan** sulfate and/or **heparin** and was completely prevented by pretreatment of erythrocytes with heparitinase, providing evidence that gC-1 mediates hemagglutination by binding to **heparan** sulfate at the cell surface. Thus, **HSV**-1-induced hemagglutination is gC-1 dependent and resembles the recently proposed mechanism by which **HSV**-1 attaches to surface **heparans** on susceptible cells, providing a simple model for initial events in the virus-cell interaction.

L9 ANSWER 23 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1993:145813 CAPLUS
DOCUMENT NUMBER: 118:145813
TITLE: Factors influencing the interaction of **herpes** simplex virus glycoprotein C with the third component of complement
AUTHOR(S): Huemer, H. P.; Larcher, C.; Dierich, M. P.; Falke, D.
CORPORATE SOURCE: Inst. Hyg., Univ. Innsbruck, Innsbruck, Austria
SOURCE: Archives of Virology (1992), 127(1-4), 291-303
CODEN: ARVIDF; ISSN: 0304-8608
DOCUMENT TYPE: Journal
LANGUAGE: English

AB The factors influencing the interaction of **herpes** simplex virus (**HSV**) glycoprotein C (gC) with the third component of complement (C3) were investigated. The ability of gC of **HSV** type 1 (gC-1) to bind to the C3b fragment of C3 was influenced by cell specific processing of gC-1 in a different manner, binding being remarkably enhanced in some cell lines following removal of sialic acid residues. Only **HSV** strains expressing gC-1 exhibited binding to C3b, even though their genome consisted mainly of **HSV**-2 sequences in some recombinants. Expression of type-2 glycoproteins gB, gD, gE, gG, gH, and gI did not alter the ability of gC-1 to bind to C3b. Rosetting of **HSV**-1 infected Vero cells with C3b-coated red blood cells (EAC) was temp. dependent and could be inhibited with purified C3b and anti-C3 antibodies. Polyanions like **heparin** or dextran sulfate were also inhibitory in a dose dependent manner, whereas C3d, neomycin and other aminoglycoside antibiotics failed to block. The complement binding function and the **heparin**-binding/attachment function of gC might be related.

L9 ANSWER 24 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1990:437675 CAPLUS
DOCUMENT NUMBER: 113:37675
TITLE: Soluble forms of **herpes** simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells
AUTHOR(S): Johnson, David C.; Burke, Rae Lyn; Gregory, Timothy
CORPORATE SOURCE: Dep. Pathol., McMaster Univ., Hamilton, ON, L8N 3Z5, Can.
SOURCE: Journal of Virology (1990), 64(6), 2569-76
CODEN: JOVIAM; ISSN: 0022-538X
DOCUMENT TYPE: Journal
LANGUAGE: English

AB **Herpes** simplex virus type 1 (**HSV**-1) and **HSV** -2 plaque prodn. was inhibited by treating cells with sol. forms of **HSV**-1 glycoprotein D (gD-1t) and **HSV**-2 glycoprotein D (gD-2t). Both glycoproteins inhibited entry of **HSV**-1 and **HSV**-2 without affecting virus adsorption. In contrast, a sol. form of **HSV**-2 glycoprotein B had no effect on virus entry into cells. Specific binding of gD-1t and gD-2t to cells was saturable, and .apprxeq.4 .times. 105 to 5 .times. 105 mols. bound per cell. Binding of gD-1t was markedly

reduced by treating cells with certain proteases but was unaffected when cell surface **heparan** sulfate glycosaminoglycans were enzymically removed or when the binding was carried out in the presence of **heparin**. Apparently, **gD** binds to a limited set of cell surface receptors which may be proteins, and these interactions are essential for subsequent virus entry into cells. However, binding of **gD** to its receptors is not required for the initial adsorption of virus to the cell surface, which involves more numerous sites (probably including **heparan** sulfate) than those which mediate **gD** binding.

L9 ANSWER 25 OF 25 CAPLUS COPYRIGHT 2003 ACS on STN
ACCESSION NUMBER: 1984:626613 CAPLUS
DOCUMENT NUMBER: 101:226613
TITLE: Binding to cells of virosomes containing **herpes** simplex virus type 1 glycoproteins and evidence for fusion
AUTHOR(S): Johnson, David C.; Wittels, Michele; Spear, Patricia G.
CORPORATE SOURCE: Dep. Microbiol., Univ. Chicago, Chicago, IL, 60637, USA
SOURCE: Journal of Virology (1984), 52(1), 238-47
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AB Envelope proteins and lipids were extd. from purified **herpes** simplex virus type 1 virions with octyl glucoside and mixed with phosphatidylcholine for prepn. of virosomes by removal of the detergent. Greater than 85% of the extd. envelope proteins, including all the glycoproteins and the nonglycosylated protein designated VP16, were assocd. with virosomes, which ranged in d. from .apprx.1.07 to 1.13 g/cm3. All the glycoproteins except gC were as susceptible to degrdn. by added protease in virosomes as in virions, indicating similar orientations in both. Approx. 30-40% of radiolabel incorporated into virosomes bound to HEp-2 cells within 1.5 h at either 4 or 37.degree.. The cell-bound virosomes were enriched for gB and deficient in other glycoproteins, in comparison with unbound or total virosomes. Binding of virosomes to HEp-2 cells could be inhibited by purified virus, **heparin**, and monospecific antiviral antibodies. Polyclonal and monoclonal anti-gB antibodies were more effective at inhibiting virosome binding than were anti-**gD** or anti-gC antibodies. Virosomes depleted of gB or **gD** did not bind to cells as efficiently as did virosomes contg. all the extd. enveloped components; this loss of binding activity was esp. pronounced on depletion of gB. The binding of **herpes** simplex virus type 1 virosomes to cells is discussed in relation to possible heterogeneity of the virosomes and comparisons with binding of virions to cells. Electron microscopic evidence that bound virosomes can fuse with the cell surface is presented.

L Number	Hits	Search Text	DB	Time stamp
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2	46234	heparin heparan herpes hsv	USPAT; US-PGPUB	2003/09/24 09:16
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5	6105	herpes hsv	EPO; DERWENT	2003/09/24 09:25
6	41	(heparin heparan) and (herpes hsv)	EPO; DERWENT	2003/09/24 09:25

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Characterization of a Heparan Sulfate Octasaccharide That Binds to Herpes Simplex Virus Type 1 Glycoprotein D*

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Herpes simplex virus type 1 utilizes cell surface heparan sulfate as receptors to infect target cells. The unique heparan sulfate saccharide sequence offers the binding site for viral envelope proteins and plays critical roles in assisting viral infections. A specific 3-*O*-sulfated heparan sulfate is known to facilitate the entry of herpes simplex virus 1 into cells. The 3-*O*-sulfated heparan sulfate is generated by the heparan sulfate D-glucosaminyl-3-*O*-sulfotransferase isoform 3 (3-OST-3), and it provides binding sites for viral glycoprotein D (gD). Here, we report the purification and structural characterization of an oligosaccharide that binds to gD. The isolated gD-binding site is an octasaccharide, and has a binding affinity to gD around 18 μM, as determined by affinity coelectrophoresis. The octasaccharide was prepared and purified from a heparan sulfate oligosaccharide library that was modified by purified 3-OST-3 enzyme. The molecular mass of the isolated octasaccharide was determined using both nanoelectrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry. The results from the sequence analysis suggest that the structure of the octasaccharide is a heptasulfated octasaccharide. The proposed structure of the octasaccharide is ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S. Given that the binding of 3-*O*-sulfated heparan sulfate to gD can mediate viral entry, our results provide structural information about heparan sulfate-assisted viral entry.

Heparan sulfates (HS),[1] highly sulfated polysaccharides, are present on the surface of mammalian cells and in the extracellular matrix in large quantities. HS play critical roles in a variety of biological interactions, including assisting viral infection, regulating blood coagulation and embryonic development, suppressing tumor growth, and controlling the eating behavior of mice by interacting with specific regulatory proteins (1–5). HS is initially synthesized as a copolymer of glucuronic acid and *N*-acetylated glucosamine by D-glucuronyl and *N*-acetyl-D-glucosaminyl transferase, followed by various modifications (6). These modifications include C_5-epimerization of glucuronic acid to form iduronic acid residues, 2-*O*-sulfation of iduronic and glucuronic acid, *N*-deacetylation and *N*-sulfation of glucosamine, as well as 6-*O*-sulfation and 3-*O*-sulfation of glucosamine. Numerous HS biosynthetic enzymes have been cloned and characterized (for review, see Esko and Lindahl (7)).

The specific sulfated saccharide sequences play critical roles in determining the functions of HS. A recent report suggests that the expression levels of various isoforms of each class of HS biosynthetic enzyme contribute to the synthesis of specific saccharide sequences in specific tissues (8). HS *N*-deacetylase/*N*-sulfotransferase, 3-*O*-sulfotransferase, and 6-*O*-sulfotransferase are present in multiple isoforms, and each isoform is believed to recognize the saccharide sequence around the modification site to generate a specific sulfated saccharide sequence (8–10). For instance, HS D-glucosaminyl-3-*O*-sulfotransferase (3-OST) isoforms generate 3-*O*-sulfated glucosamine that is linked to different sulfated uronic acid residues. 3-OST-1 transfers sulfate to the 3-OH position of the *N*-sulfated glucosamine residue that is linked to a glucuronic acid residue at the nonreducing end (GlcUA-GlcNS ± 6S), whereas, 3-OST-3 transfers sulfate to the 3-OH position of the *N*-unsubstituted glucosamine residue that is linked to a 2-*O*-sulfated iduronic acid at the nonreducing end (IdoUA2S-GlcNH$_2$ ± 6S) (11). The differ-

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[1] The abbreviations used are: HS, heparan sulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; nESI-MS, nanoelectrospray ionization mass spectrometry; HSV-1, herpes simplex virus type 1; gB, gC, and gD, herpes envelope glycoprotein B, glycoprotein C, and glycoprotein D, respectively; 3-OST; heparan sulfate D-glucosaminyl-3-*O*-sulfotransferase; ΔUA, $\Delta^{4,5}$-unsaturated uronic acid; GlcUA, D-glucuronic acid; IdoUA, α-iduronic acid; GlcNH$_2$, *N*-unsubstituted glucosamine; MWCO, molecular weight cut-off; AnMan, 2,5-anhydromannitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography.

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ence in substrate specificity of 3-OSTs results in distinct biological functions of the HS modified by 3-OSTs. For example, HS modified by 3-OST-1 binds to antithrombin and has anticoagulant activity (12), whereas the HS modified by 3-OST-3 binds to herpes simplex 1 envelope glycoprotein D (gD) and assists in viral entry (13).

Herpes simplex virus type 1 (HSV-1) is a member of the herpesvirus family, and infection in humans is prevalent. HSV-1 infection requires a two-step process that can be separated experimentally: attachment to cells and entry into cells (14). It is now known that HS is involved in assisting viral binding as well as viral entry (15). HSV-1 binds to host cells through an interaction of virion envelope glycoprotein C (gC), or in some cases of glycoprotein B (gB), with HS (16–18). Structural analysis of gC-binding HS revealed that a minimum of 10–12 sugar residues containing IdoUA2S and GlcNS(or Ac)6S are necessary (19), and this conclusion was confirmed by another study (20).

A recent report suggests that a specific 3-*O*-sulfated HS is involved in assisting HSV-1 entry (13). The 3-*O*-sulfated HS is generated by 3-OST-3, but not by 3-OST-1. It should be noted that 3-OST-3-modified HS is rarely found in HS from natural sources, suggesting that HSV-1 recognizes a unique saccharide structure (11). In addition, a biochemical study revealed that 3-*O*-sulfated HS provides binding sites for HSV-1 envelope glycoprotein gD, which is a key viral protein involved in entry of HSV-1. It is believed that the interaction between gD and the 3-*O*-sulfated HS triggers the fusion between the virus and the cell in the presence of other viral envelope proteins, including gB, gH, and gL, via an uncharacterized mechanism. The study of the crystal structure of gD and herpes entry receptor HveA suggest that the binding of HveA to gD induces conformational changes in gD (21). This study also predicts a 3-*O*-sulfated HS-binding pocket on gD near the HveA-binding site (21). The exact carbohydrate sequence of the gD-binding site in 3-*O*-sulfated HS remains to be investigated.

In this article, we report the characterization of the structure of a gD-binding octasaccharide. The results from extensive sequencing analysis suggest that the structure of the gD-binding octasaccharide is ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S (residue 1 is GlcNH$_2$3S6S and residue 8 is ΔUA). The octasaccharide apparently has two motifs: a relatively low sulfation domain (residues 5–8) that contains two sulfate groups and a high sulfation domain (residue 1 to residue 4) that contains five sulfate groups. Although we still do not know the contribution of each sulfate group to the binding affinity of the octasaccharide and gD, the results from this study will provide the structural information to understand HS-assisted viral infection mechanisms.

EXPERIMENTAL PROCEDURES

Materials

Recombinant 3-OST-3A and 3-OST-1 enzymes were expressed in Sf9 cells using baculovirus expression system. The enzymes were purified by using heparin-Toyopearl and 3′,5′-ADP-agarose chromatographies as described previously (11, 22). [^{35}S]PAPS was prepared by incubating 0.4 mCi/ml [^{35}S]Na_2SO_4 (carrier-free, ICN) and 16 mM ATP with 5 mg/ml dialyzed yeast extract (Sigma) (12). Iduronate-2-sulfatase, α-iduronidase, α-*N*-acetylglucosaminidase, glucosamine-6-sulfatase, and sulfamidase were obtained from Glyko. Recombinant heparin lyase I (EC 4.2.2.7), II (no EC number), and III (E.C. 4.2.2.8) were prepared as described previously (23). $\Delta^{4,5}$-Glycuronidase was isolated from *Flavobacterium heparinum* (24). HS from bovine kidney was obtained from ICN. A truncated form of herpes simplex virus 1 glycoprotein D, gD-1(306t), and monoclonal anti-gD-(DL6) were prepared as previously described (25).

Preparation of gD-binding Octasaccharide

Preparation of the HS Oligosaccharide Library—The library was prepared by incubating HS with limited amounts of heparin lyase III followed by size fractionation on a Bio-Gel P-6 (Bio-Rad) column as described by Pye *et al.* (26). In a typical preparation, HS from bovine kidney (1 mg) was incubated with 2 milliunits of heparin lyase III in 1 ml of buffer containing 50 mM sodium phosphate and 100 μg/ml bovine serum albumin, pH 7.0, at 37 °C overnight. The digestion was terminated by heating at 100 °C for 15 min. The sample was then loaded on a Bio-Gel P-6 (0.75 × 200 cm) equilibrated with 0.5 M ammonium bicarbonate at a flow rate of 5 ml/h and 0.5-ml fractions were collected. The absorbance at 232 nm was measured for each fraction. Peaks corresponding to tetra- to greater than dodecasaccharides were pooled individually, and dialyzed against 50 mM ammonium bicarbonate using MWCO 3,500 membrane. Each pool was dried on a Speed-Vac concentrator (Labconco) and reconstituted in 50 μl of water. The optical density (232 nm) of the resultant solution was about 3. We processed a total of 40 mg of HS to obtain a sufficient amount of gD-binding octasaccharide for the structural analysis.

Preparation of 3-O-Sulfated Oligosaccharides—To prepare 3-OST-3A-modified HS oligosaccharide, 20 μl of the oligosaccharide library or intact HS (1 μg) was mixed with 240 ng of purified 3-OST-3A enzyme and 10 μM [^{35}S]PAPS (14,000 dpm/pmol) in a buffer containing 50 mM MES, 1% Triton X-100, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 150 mM NaCl, and 168 μg/ml bovine serum albumin, pH 7, in a final volume of 50 μl. The reaction was incubated at 37 °C for 2 h and was then heated at 100 °C for 2 min. The resultant solution was centrifuged at 14,000 rpm for 1 min to remove insoluble materials. The supernatant was dialyzed against 50 mM ammonium bicarbonate using MWCO 3,500 membrane and dried. To prepare 3-OST-1-modified oligosaccharides, we followed nearly identical procedures except for omitting the 150 mM NaCl and using 70 ng of 3-OST-1 enzyme during the enzymatic modification reaction.

Purification of the 3-O-Sulfated Octasaccharides by HPLCs—The 3-OST-3-modified oligosaccharides were applied to a silica-based polyamine (PAMN) HPLC column (0.46 × 25 cm, Waters). The column was eluted with a linear gradient of KH_2PO_4 from 350 mM to 1 M for 60 min followed by an additional wash with 1 M KH_2PO_4 for 20 min at a flow rate of 1 ml/min (11). The fractions containing ^{35}S-radioactivity were pooled separately and resolved on Bio-Gel P-6. The fractions were dialyzed against 25 mM ammonium acetate using MWCO 3,500 membrane and dried. They were further purified by DEAE-NPR HPLC chromatography (0.46 × 7.5 cm, Tosohaas). The DEAE-NPR column was eluted with a linear gradient of NaCl in 50 mM Tris-HCl, pH 7, from 100 to 500 mM in 60 min followed by an additional wash for 20 min with 1 M NaCl in 50 mM Tris-HCl, pH 7, at a flow rate of 0.5 ml/min. The eluted oligosaccharides were monitored by ^{35}S-radioactivity and the absorbance at 232 nm. We obtained about 200 to 300 pmol of purified ^{35}S-labeled oligosaccharides from 20 mg of HS that was partially digested with heparin lyase III. We did observe a UV peak that overlapped with the ^{35}S-radioactive peak when a large amount of 3-*O*-^{35}S-sulfated oligosaccharides (>200 pmol) were injected on DEAE-NPR-HPLC.

Determination of the Binding of 3-O-Sulfated HS Oligosaccharides to gD—The assay for determining the binding of 3-*O*-sulfated HS oligosaccharides to gD was carried out by an immunoprecipitation procedure using gD and anti-gD monoclonal antibody as described previously but at a lower pH (13). Briefly, 3-*O*-sulfated HS (1–10 pmol) was incubated in 50 μl of buffer containing 50 mM MES and 0.01% Triton, pH 6 (binding buffer), and 2 mg/ml gD at room temperature for 30 min. The anti-gD monoclonal antibody DL6 (5 μl) was added and incubated at 4 °C for 1 h followed by addition of the protein A-agarose gel (80 μl of 1:1 slurry) and agitated at 4 °C for an additional hour. The protein A-agarose gel (Pierce) was then washed with 0, 50, 150, and 500 mM NaCl in the above binding buffer.

The binding affinity between 3-*O*-sulfated oligosaccharides and gD was determined using affinity co-electrophoresis, as previously described (13). The gel was dried and analyzed on a PhosphorImager (Amersham Biosciences, Storm 860) to determine the migration of [^{35}S]oligosaccharides. The ^{35}S-intensity was plotted against the migration distance through the separation zone to define the distance migrated in the presence or absence of gD.

Determination of the Structure of a gD-binding Octasaccharide

Enzymatic and Nitrous Acid Degradation of Octasaccharides—The conditions for digestion with $\Delta^{4,5}$-glycuronidase and HS glycuronate-2-sulfatase were described elsewhere (27). The conditions for the nitrous

acid degradations under pH 1.5 and 4.5 were described in a prior publication (8). The degraded octasaccharide was analyzed by DEAE-NPR-HPLC.

N-Acetylation of Oligosaccharides—The oligosaccharide (5×10^5 to 1×10^6 cpm, 36–72 pmol) was dissolved in 20 μl of a solvent containing *N',N'*-dimethylformamide and triethylamine (1:1, v/v) and 5 μl of acetic anhydride, and incubated on ice for 1 h. Tris (20 μl of 50 mM) was then added and the reaction mixture was incubated on ice for an additional hour. The sample was then diluted with 10 volumes of water and dialyzed against 50 mM ammonium bicarbonate using a MWCO 3,500 membrane.

Derivatizations—Derivatizations were carried out by reacting 5 μl of oligosaccharide solution with 5 μl of 50 mM semicarbazide and 60 mM Tris acetic acid (pH 7.0, prepared fresh daily) for 16 h at 30 °C.

Analysis of HS Oligosaccharides Using Mass Spectrometry—Two mass spectrometry techniques, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and nanoelectrospray ionization mass spectrometry (nESI-MS), were employed. The MALDI-MS spectra were acquired in the linear mode using a Perseptive Biosystems Voyager Elite reflectron time-of-flight instrument fitted with a 337-nm laser as described elsewhere (28). The nESI-MS analysis was carried out using a Micromass Quattro II with QhQ geometry, a Z-spray source, and pulled borosilicate glass nanovials (29). In the neutral loss scan, MS/MS spectra were obtained by scanning Q1 and Q3 with an offset of 26.7 or 20 m/z in their scan cycles, corresponding to the loss of sulfate from the triple or quadruple charged octasaccharide, respectively (29). To obtain a high quality nESI-MS spectrum, the purified octasaccharide was further dialyzed against 25 mM ammonium acetate (purity of ammonium acetate is 99.9999%, Aldrich) using MWCO 13,000 hollow fiber dialysis tubing (Spectrum). Control studies showed that 80–95% of 3-*O*-[^{35}S]pentasaccharide (M_r = 1507) could be recovered using this dialysis tubing.

Analysis of Oligosaccharides by Capillary Electrophoresis—The approach for the analysis of oligosaccharides followed a previously described method with modifications (30). Briefly, the analysis was carried out on a Beckman P/ACE MDQ unit using an uncoated fused silica capillary (inner diameter = 75 μm; L_{tot} = 106 cm). Hydrodynamic injection was employed under 9.5 p.s.i. for 5 s. About 274 nl of the sample was calculated to be injected by *CE Expert* software. The electrolyte was a solution of 10 μM dextran sulfate and 50 mM Tris-phosphoric acid, pH 2.5. Separation was carried out at 25 kV.

TABLE I
The binding of 3-O-sulfated oligosaccharides to gD

The binding of HS and oligosaccharides to gD was carried out at pH 6 by using an immunoprecipitation approach as described under "Experimental Procedures."

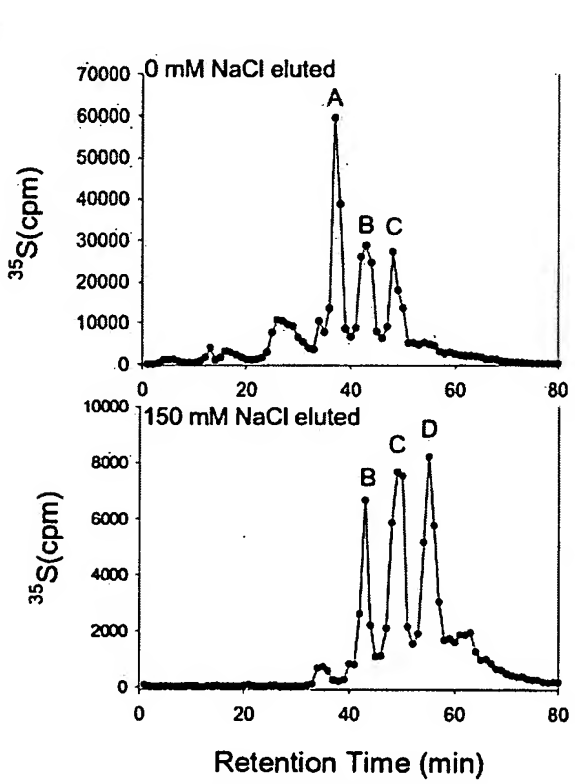
Size of the oligosaccharides	gD-binding	
	3-OST-1-modified	3-OST-3A-modified
	%	
Intact HS	8.8	22.9
>Dodecasaccharides	2.0	7.2
Dodecasaccharides	2.4	7.6
Decasaccharides	5.3	7.0
Octasaccharides	1.3	5.4
Hexasaccharides[a]	Not determined	3.4

[a] Because 3-OST-1 sulfated hexasaccharides very poorly, we could not obtain sufficient amount of 3-OST-1-modified hexasaccharides for the binding experiment. Therefore, we were unable to compare the binding of 3-OST-1-modified hexasaccharides and 3-OST-3A-modified hexasaccharides. Nevertheless, a gD-binding octasaccharide was isolated from the 3-OST-3 modified-hexasaccharide library as described in the text.

RESULTS

Isolation of the gD-binding Octasaccharide

A gD-binding octasaccharide was purified from a 3-OST-3A-modified HS oligosaccharide library. The HS oligosaccharide library was prepared by incubating HS with a limited amount of heparin lyase III. The resultant material was fractionated by a Bio-Gel P-6 column based upon the size of the oligosaccharides, obtaining di-, tetra,-, . . . , dodeca-, and >dodecasaccha-



B

The binding of the purified 3-*O*-[^{35}S] sulfated oligosaccharides and gD

Samples	gD-binding (%)
Fraction A	9.1± 3% (n=2)
Fraction B	10.8%
Fraction C	11.4%
Fraction D	32.4 ± 6% (n=6)

FIG. 1. **Purification of a gD-binding oligosaccharide by PAMN-HPLC.** *Panel A* shows the profiles of the 3-*O*-^{35}S-sulfated oligosaccharides eluted from a protein A-agarose column with different concentrations of NaCl. The *top chromatogram* is the profile of the fractions that were eluted without sodium chloride; the *bottom chromatogram* is the profile of the fractions that were eluted with 150 mM NaCl. *Panel B* shows the binding of purified oligosaccharides and gD using an immunoprecipitation approach.

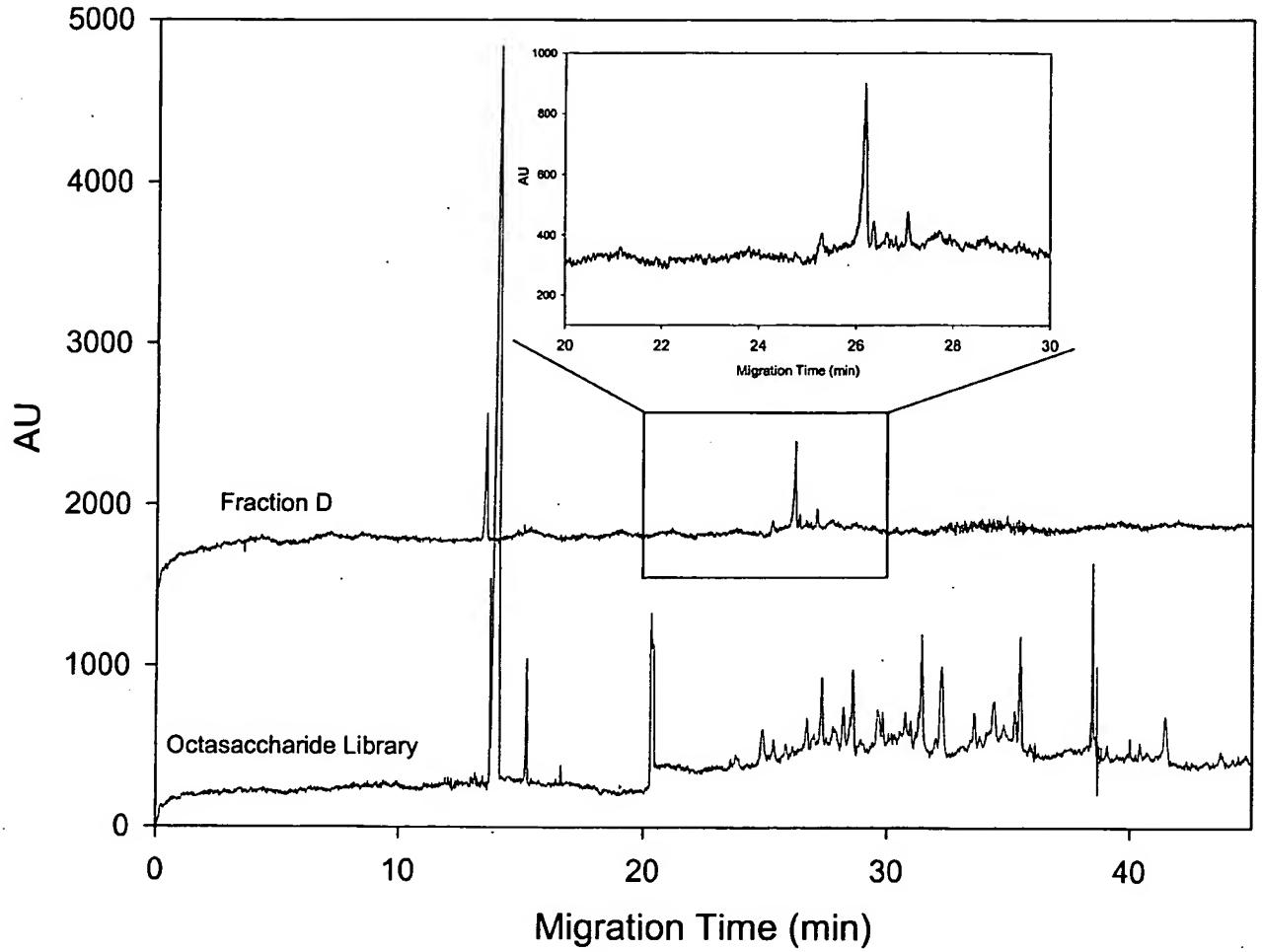


FIG. 2. **The electrophoretogram of fraction D analysis with capillary electrophoresis.** Purified fraction D was analyzed on capillary electrophoresis with an on-line UV detector at 230 nm under reverse polarity conditions. The *bottom electrophoretogram* shows the separation of HS octasaccharide library. The *top electrophoretogram* shows the separation of fraction D. The *inset* shows the enlarged region where fraction D migrated.

rides (data not shown), and a similar approach to prepare a HS oligosaccharide library was reported by Pye and colleagues (26). Because of the limited resolution capability of Bio-Gel P-6, each oligosaccharide library undoubtedly contained the oligosaccharides with different sizes. These fractions were then subjected to 3-OST-3A modification and assayed for gD binding (Table I). Because 3-OST-1-modified HS does not bind to gD, we utilized the 3-OST-1-modified oligiosaccharides as a negative control (13). As shown in Table I, the gD-binding percentage of 3-OST-3A-modified oligosaccharides was about 3-fold higher than that of the 3-OST-1-modified counterparts. We chose to purify a gD-binding oligosaccharide from the 3-OST-3A-modified hexasaccharide pool based upon the following two reasons: 1) the purification of hexasaccharides or octasaccharides can be achieved by anion exchange HPLC; 2) sequencing analysis for hexa- or octasaccharide is significantly less complex than larger oligosaccharides.

We purified a gD-binding oligosaccharide by successive anion-exchange HPLC, PAMN-, and DEAE-NPR-HPLC. Five major 3-*O*-^{35}S-sulfated oligosaccharides were resolved by PAMN-HPLC (data not shown). To identify which [^{35}S]oligosaccharide has the highest binding affinity for gD, the 3-OST-3A-modified oligosaccharides were fractionated by an immunoprecipitation approach as described under "Experimental Procedures." The eluents were analyzed by PAMN-HPLC (Fig. 1*A*). Comparing the chromatograms of the 3-*O*-^{35}S-sulfated oligosaccharides eluted from protein A-agarose under different concentrations of sodium chloride, we found that fraction D was present when the protein A-agarose was eluted with 150 mM NaCl (Fig. 1*A*, *bottom chromatogram*). In contrast, fraction A was present when the protein A-agarose was eluted with the buffer without NaCl (Fig. 1*A*, *top chromatogram*). This result suggests that fraction D has higher affinity for gD than fraction A. As indicated, it was observed that 32% of fraction D binds gD, whereas only 9% of fraction A binds to gD (Fig. 1*B*). Thus, we designated fraction D as a gD-binding oligosaccharide and fraction A as a gD-nonbinding oligosaccharide. Fraction B and fraction C were considered gD-nonbinding oligosaccharides, and were not subject to further structural study as their binding percentages to gD are similar to that of fraction A (Fig. 1*B*). Additional ^{35}S-labeled molecules were eluted from the protein A-agarose column with 500 mM sodium chloride. However, those molecules did not give sharp peaks on PAMN-HPLC. In addition, those molecules migrated as the oligosaccharides that were much larger than octasaccharides on Bio-Gel P-6. It is possible that these molecules represented the ^{35}S-labeled oligosaccharide contaminants that were larger than octasaccharides in the oligosaccharide library. Fraction D was further purified on DEAE-NPR-HPLC.

To confirm the purity, fraction D was analyzed by capillary electrophoresis using a UV 230 nm on-line detector. As shown in Fig. 2 (*bottom electrophoretogram*), the octasaccharide li-

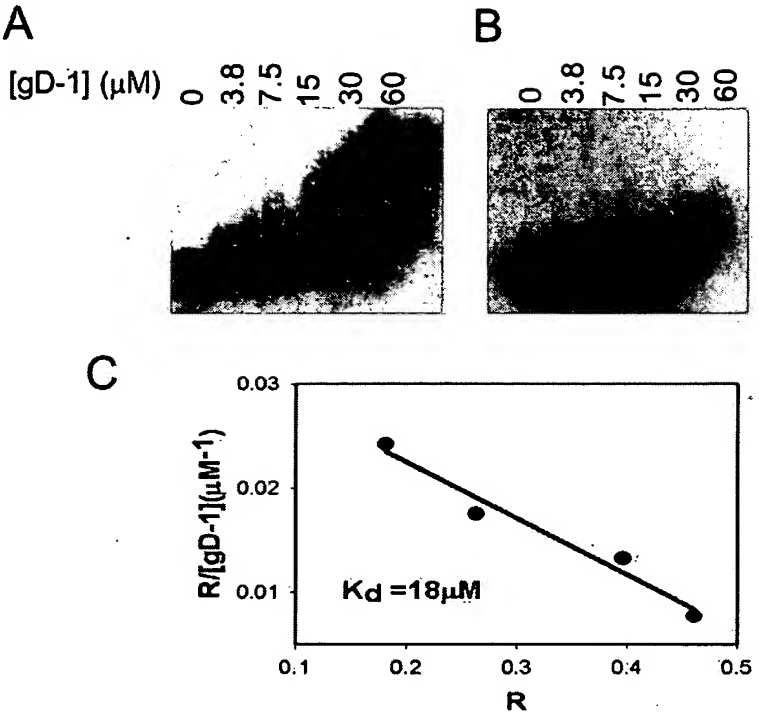


FIG. 3. **Binding constant (K_d) for the interaction between fraction D and gD-1.** *Panel A* presents the autoradiograph of the agarose gel in which purified fraction D was subjected to electrophoresis through zones containing gD-1 at the concentrations indicated. Approximately 30,000 cpm (3×10^{-12} mol)/lane of [^{35}S]fraction D was loaded in each separation zone. *Panel B* presents the autoradiograph of the agarose gel in which purified [^{35}S]fraction A (15,000 cpm, 1.5×10^{-12} mol/lane) was subjected to electrophoresis through zones containing gD at the concentrations indicated. *Panel C* represents the plot of $R/[gD]_{total}$ *versus* R, where the retardation coefficient, $R = (M_0\text{-}M)/M_0$. M_0 is the migration of free [^{35}S]fraction D, and M is the observed migration of [^{35}S]fraction D in the presence of gD-1. Assuming that [^{35}S]fraction D and gD-1 form a 1:1 complex and gD-1 is in great excess, this plot should yield a straight line with a slope of $-1/K_d$ according to the Scatchard equation. The linear coefficient value of the plot is 0.98, and the calculated K_d is 18 μM. Because there was no obvious retardation for fraction A under the assay conditions, we were unable to perform a graphical analysis to determine the binding constant (K_d) between gD and fraction A.

brary was well resolved by capillary electrophoresis, suggesting that the resolution of the oligosaccharides on capillary electrophoresis is high. Fraction D migrated predominantly as a single peak under such conditions (Fig. 2, *top electropherotogram*). In addition, the area of the major UV peak is consistent with the estimated concentration of the octasaccharide based upon the specific ^{35}S-radioactivity. Having considered the minor UV peaks resulting from contaminants, we calculated the purity of fraction D to be greater than 80%.

We also determined the binding affinity (K_d) between fraction D and gD using affinity coelectrophoresis as described by Lee and Lander (31). Fraction D was separated under electrophoresis in an agarose gel through zones containing gD at various concentrations (Fig. 3*A*). From these data, the K_d for fraction D and gD was determined to be 18 μM (Fig. 3*C*), which is somewhat higher than the K_d of intact 3-*O*-sulfated HS and gD (2 μM) (13). We also attempted to determine the K_d between fraction A (gD-nonbinding oligosaccharide) and gD using this method. We failed to observe any obvious retarded migration of fraction A, suggesting that the binding affinity between fraction A and gD is low (Fig. 3*B*). We estimated that the K_d for fraction A and gD is greater than 200 μM.

Structural Characterization of Fraction D

Analysis of Fraction D by nESI-MS and MALDI-MS—The molecular mass of fraction D was determined by both nESI-MS and MALDI-MS. The nESI-MS spectrum of fraction D is shown in Fig. 4. The sample shows a triple charged ion, $[M\text{-}3H]^{3-}$, at *m/z* 648.8 and a strong quadruple charged ion, $[M\text{-}4H]^{4-}$, at *m/z* 486.4 (Fig. 4*A*). We confirmed that the signals at *m/z* 648.8 and 486.4 contain sulfate groups by using neutral loss experiments as described in a prior publication (29). Briefly, the isolated ions were sequentially admitted to a collision cell filled with argon under controlled energy conditions, resulting in limited dissociation. The linkage between the sulfate and the hydroxyl group is labile, and the products from a series of sulfate losses within the collision cells are common. The experiments were designed to detect molecular ions that lose 20 *m/z* (corresponding to the loss of the sulfate from the quadruple charged oligosaccharide), and signals were detected 486.4, 491.9, and 495.9 *m/z* (Fig. 4*B*). Similarly, neutral loss scans looked for oligosaccharides that lose 26.7 *m/z* (corresponding to the loss of the sulfate from the triple charged oligosaccharide), and signals were detected at 648.9, 656.0, and 661.4 *m/z* (Fig. 4*C*). Taken together, these data suggest that the signals at 648.8 and 486.4 *m/z* were likely derived from triple and quadruple charged oligosaccharides, respectively. From these data, the molecular mass of fraction D was calculated to be 1949.5 Da (from the triple charged ion, the molecular mass of fraction D is $648.8 \times 3 + 3 = 1949.4$; from the quadruple charged ion, the molecular mass of fraction D is $486.4 \times 4 + 4 = 1949.6$). The determined molecular mass for fraction D was very close to the theoretical value (1950.1 Da) for a heptasulfated octasaccharide with one *N*-acetylated glucosamine residue ($\Delta UA(UA)_3(GlcN)_3\ GlcNAc(SO_3H)_7$, $C_{50}H_{78}O_{62}N_4S_7$).[2]

We also determined the molecular mass of fraction D by using MALDI-MS. MALDI-MS requires that a complex between fraction D and a synthetic peptide $(Arg\text{-}Gly)_{19}\text{-}Arg$ be formed (32). After subtracting the contribution of the protonated peptide, the molecular mass of fraction D was calculated to be 1951.2 Da (spectrum not shown).[3] Thus, the result of MALDI-MS is consistent with the result of nESI-MS.

Sequencing Analysis of Fraction D—Because we had demonstrated that fraction D is a heptasulfated octasaccharide with an *N*-acetylated glucosamine residue, we conducted sequencing analysis to identify the position of the sulfate groups in each residue and to determine the identity of uronic acid residues (*i.e.* glucuronic acid or iduronic acid).

[^{35}S]Disaccharide Analysis of Fraction D—A disaccharide analysis was performed to determine the identity of the disaccharide with a 3-*O*-[^{35}S]sulfate group. In this experiment, frac-

[2] We calculated the molecular weight of fraction D based on ^{32}S, because [^{35}S]sulfate represents less than 0.4% of total 3-*O*-sulfation.

[3] The molecular mass of fraction A was determined to be 1834 Da by MALDI-MS. The molecular mass is consistent with an octasaccharide with five sulfate groups and two *N*-acetylated glucosamine residues.

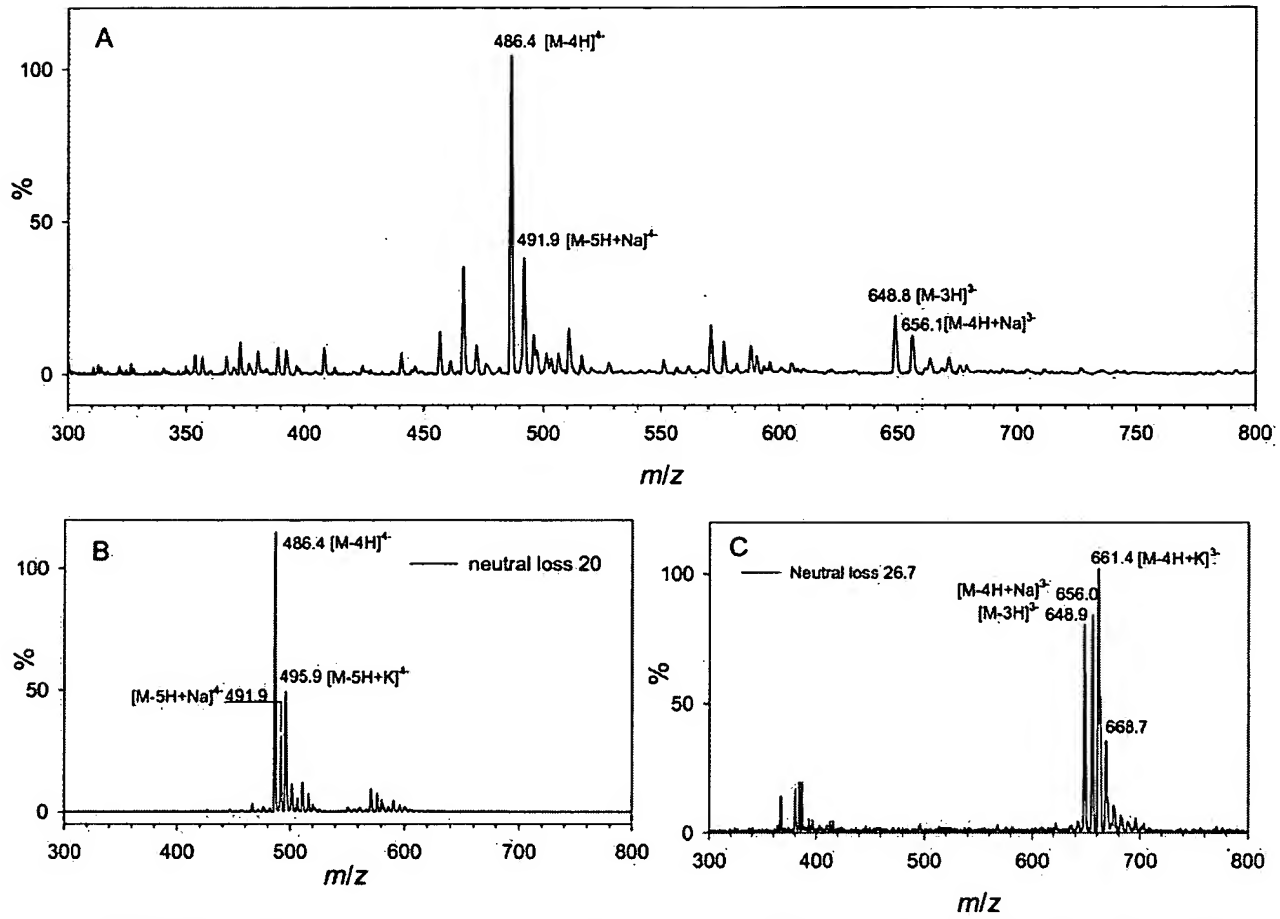


FIG. 4. **nESI-MS spectra of fraction D.** The mass spectrum of fraction D is shown in *panel A*. A neutral loss scan for the loss of 20 *m/z* is shown in *panel B*. A neutral loss scan for the loss of 26.7 *m/z* is shown in *panel C*.

tion D was degraded with nitrous acid at pH 1.5 followed by sodium borohydride reduction. The resultant disaccharides were analyzed by reverse-phase ion pairing HPLC (11). We found that nearly 90% of the [^{35}S]disaccharide was IdoUA2S-[3-^{35}S]AnMan3S6S (Fig. 5).[4] The result suggests that the 3-*O*-^{35}S-sulfation site is present in a disaccharide with a structure of -IdoUA2S-GlcNH$_2$3S6S-, provided that a previous report (11) demonstrated that 3-OST-3A sulfates an *N*-unsubstituted glucosamine residue.

Sequencing Analysis from the Reducing End—Fraction D was reacted with semicarbazide to form a semicarbazone at the reducing end, as illustrated in Fig. 6*C* (30). This reaction increases the molecular mass of the oligosaccharide by 56.1 Da. The semicarbazone moiety serves as a mass tag during sequence analysis to differentiate oligosaccharides derived from the reducing end of the parent compound. By capillary electrophoresis, we confirmed that greater than 95% of Fraction D was labeled under the standard derivatization conditions (data not shown). The molecular mass of the derivatized fraction D was determined to be 2008 Da using MALDI-MS (Fig. 6*A*), confirming that the tag was present. Upon treatment of the derivatized oligosaccharide with heparin lyase II, a tetrasaccharide of mass 1131.1 was observed (Fig. 6*B*). This mass corresponds to a pentasulfated tetrasaccharide labeled with the mass tag (1075.0 + 56.1 Da). By capillary electrophoresis, this tetrasaccharide had an identical migration time to the standard ΔUA2S-GlcNS-IdoA2S-GlcNH$_2$3S6S-semicarbazide (11) (data not shown). We further strengthened the conclusion by demonstrating that residue 4 (ΔUA2S) carries the 2-*O*-sulfate group as described below.

We examined the susceptibility of the reducing end tetrasaccharide (residues 1–4) to HS glycuronate-2-sulfatase (Fig. 7). The reducing end tetrasaccharide was prepared by subjecting the octasaccharide to digestion by heparin lyase II, because it was reported that the tetrasaccharides containing a 3-*O*-sulfated glucosamine residue is resistant to digestion (11, 33). Comparing the elution times of the undigested and digested reducing end tetrasaccharide, we found that the tetrasaccharide was susceptible to HS glycuronate-2-sulfatase digestion. Thus, the reducing end residue (residue 4) of the tetrasaccharide is a 2-*O*-sulfated $\Delta^{4,5}$-uronic acid, provided that HS glycuronate-2-sulfatase specifically reacts with this residue (24).[5] Taken together, these results demonstrate that fraction D contains a tetrasaccharide with a structure of ΔUA2S-GlcNS-

[4] Two minor [^{35}S]peaks were also observed, representing [^{35}S]sulfate and IdoUA2S-AnMan3S, respectively. The IdoUA2S-AnMan3S is probably the partial desulfated IdoUA2S-AnMan3S6S because of nitrous acid degradation or the product of the minor contaminants in the fraction D.

[5] It should be noted that we identified the positions of five sulfate groups in the reducing end tetrasaccharide to this end. Because low-pH nitrous, pH 1.5, degradation of fraction D resulted in a trisulfated disaccharide (IdoUA2S-AnMan3S6S) (Fig. 5), the octasaccharide must contain a trisaccharide carrying four sulfate groups with a structure of –GlcNS-IdoUA2S-GlcNH$_2$3S6S–. Another sulfate group is located at the 2-OH position of residue 4.

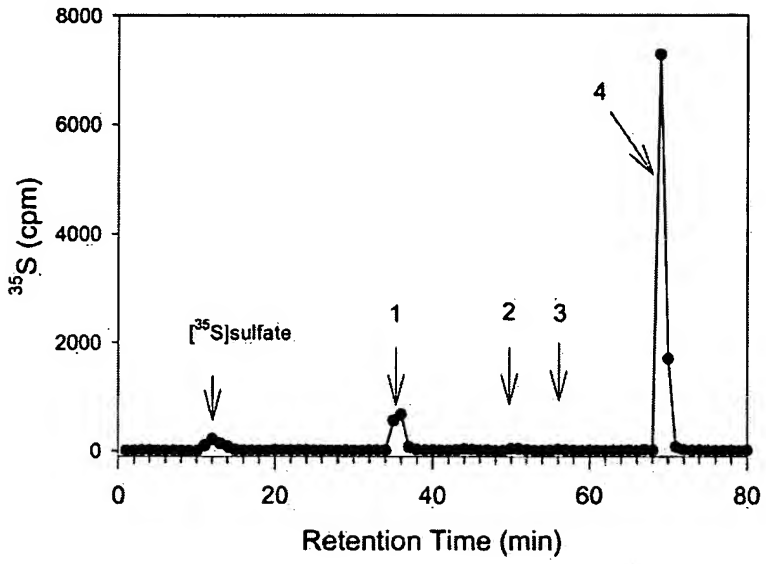


FIG. 5. **RPIP-HPLC chromatogram of low-pH nitrous acid degraded fraction D.** Fraction D was treated with nitrous acid at pH 1.5 followed by sodium borohydride reduction. The resultant disaccharides were resolved on RPIP-HPLC. *Arrows* indicate the elution positions of the disaccharide standards. *1* represents IdoUA2S-AnMan3S; *2* represents GlcUA-AnMan3S6S; *3* represents IdoUA2S-AnMan6S; and *4* represents IdoUA2S-AnMan3S6S.

IdoUA2S-GlcNH$_2$3S6S; the location of this tetrasaccharide is from residue 1 to 4.[6]

Sequencing Analysis from the Nonreducing End—Sequencing from the nonreducing end was accomplished by using various exoenzymes followed by chromatography on DEAE-NPR-HPLC. The goal of the nonreducing sequencing analysis was to identify the position of the two remaining sulfate groups within residues 5–8, given that five sulfate groups were determined to be present on residues 1–4.

The sequencing strategy from the nonreducing end and the experimental data are shown in Fig. 8. The retention time of fraction D was shifted from 46 to 38 min after digestion with HS $\Delta^{4,5}$-glycuronidase (Fig. 8*B*). This result suggests that a $\Delta^{4,5}$-unsaturated uronic acid residue was at the nonreducing end, based upon the substrate specificity of HS glycuronidase (24, 34). The minor unknown product was removed prior to the sulfamidase digestion. The resultant heptasaccharide was susceptible to digestion by sulfamidase, as observed by a shift in the retention time from 38 to 30 min on DEAE-NPR-HPLC (Fig. 8*C*), suggesting that it contains an *N*-sulfated glucosamine residue. The sample was then subjected to *N*-acetylation by incubating with acetic anhydride to generate an *N*-acetylated glucosamine residue at the nonreducing end, because α-*N*-acetylglucosaminidase does not react with an *N*-unsubstituted glucosamine. A successful *N*-acetylation on the *N*-unsubstituted glucosamine residue of the heptasaccharide after treatment with acetic anhydride was confirmed, as a shift in the retention time on DEAE-NPR-HPLC was observed (Fig. 8*D*). The *N*-acetylated heptasaccharide was susceptible to α-*N*-acetylglucosaminidase digestion as a shift of retention time from 33 to 38 min was observed (Fig. 8*E*).[7] At this point, fraction D was converted to a hexasaccharide carrying six sulfate groups. The undigested product was removed after purification by DEAE-NPR-HPLC. The resultant hexasaccharide was susceptible to iduronate-2-sulfatase digestion as the retention time was shifted from 38 to 27 min after treatment (Fig. 8*F*). Furthermore, the resultant oligosaccharide was susceptible to α-iduronidase digestion (Fig. 8*G*). The results from the susceptibilities to digestions by iduronate-2-sulfatase and α-iduronidase suggest that residue 6 is a 2-*O*-sulfated iduronic acid residue. The positions of all sulfate groups were therefore determined. The proposed structure is: ΔUA-GlcNS-IdoUA2S-GlcNAc-GlcUA2S (or IdoUA2S)-GlcNS-IdoUA2S-GlcNH$_2$3S6S.

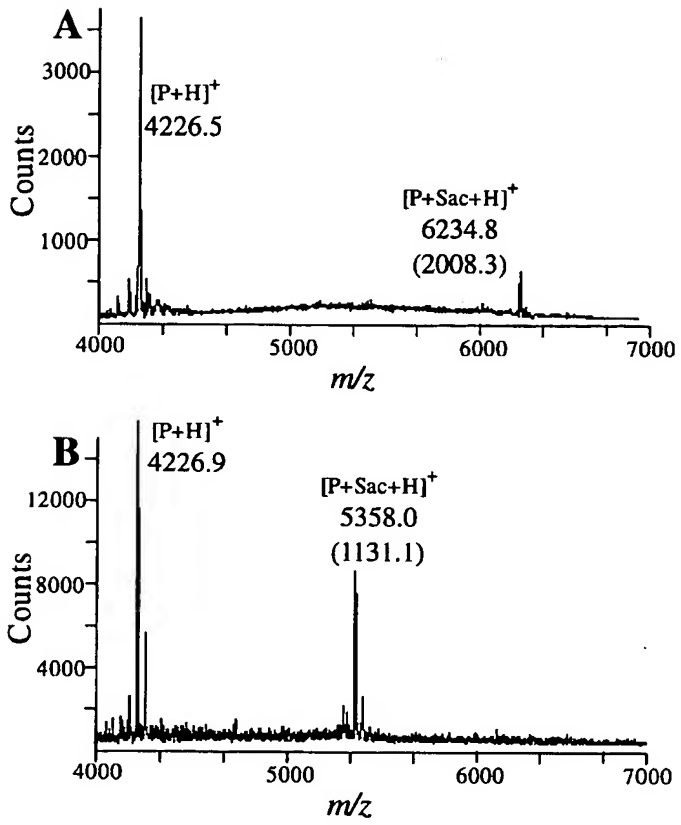
To further confirm the results from nonreducing sequencing analysis, fraction D was treated with heparin lyase II and analyzed by capillary electrophoresis. A tetrasaccharide (residues 1–4) and two disaccharides were generated after digestion as the tetrasaccharide carrying the 3,6-disulfated glucosamine was not susceptible to digestion by heparin lyase II as described above. Enzyme-produced tetrasaccharide coeluted with a tetrasaccharide standard with a structure of ΔUA-GlcNS-IdoUA2S-GlcNH$_2$6S3S (corresponding residues 1–4, data not shown). The preparation of this tetrasaccharide standard was published previously (11). In addition, two resultant disaccharides were identified to be ΔUA-GlcNS and ΔUA2S-GlcNAc (data not shown). Again, the results from heparin lyase II digestion are consistent with the proposed structure for fraction D.

We attempted to determine the identity of residue 4. As described above, we concluded that residue 4 carries a 2-*O*-sulfate group. To address the identity of residue 4, a tetrasaccharide, representing residues 1–4, was prepared by deacetylation followed by nitrous acid degradation at pH 4.5.[8] The resultant tetrasaccharide, which carried a 2-*O*-sulfated uronic

[6] We also attempted to prove the presence of an *N*-unsubstituted glucosamine residue at the reducing end by treating the octasaccharide with nitrous acid at pH 4.5 followed by sodium borohydride reduction. The molecular mass of the resultant octasaccharide was determined using nESI. We found that the molecular mass of the high pH nitrous-treated octasaccharide is 1855 Da, a loss of 95 Da. A reduction of 95 Da is consistent with the deamination (−15 Da) and a loss of sulfate (−80 Da). Our result suggested that desulfation also occurred during nitrous acid, pH 4.5, treatment or during analysis of nESI-MS. Nevertheless, the result is consistent with the fact that the octasaccharide contains an *N*-unsubstituted glucosamine residue.

[7] We observed that only 50% of the heptasaccharide was digested by α-*N*-acetylglucosaminidase. A similar incomplete digestion was observed for a pentasaccharide with a structure of GlcNAc-GlcUA-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6SOMe digested by α-*N*-acetylglucosaminidase. The pentasaccharide was prepared by incubating acetic anhydride with a pentasaccharide with a structure of GlcNH$_2$-GlcUA-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6SOMe. The latter pentasaccharide was generated from GlcNS6S-GlcUA-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6SOMe (29) by sequential digestions by glucosamine-6-sulfatase and sulfamidase.

[8] The tetrasaccharide was prepared by incubating purified fraction D with hydrazine for deacetylation. The deacetylated octasaccharide was degraded by nitrous acid at pH 4.5 and sodium borohydride reduction. The resultant oligosaccharide migrated as a tetrasaccharide on Bio-Gel P-6, and was purified by DEAE-NPR HPLC. The proposed structure of this tetrasaccharide is UA2S-GlcNS-IdoUA2S-AnMan3S6S.



C

ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S

(MW = 1950 Da)

Semicarbazide

ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S—Semicarbazide

(MW = 2006 Da, measured MW = 2008 Da)

Heparin lyase II digestion

Disaccharides

ΔUA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S—Semicarbazide

(MW = 1130 Da, measured MW = 1131 Da)

FIG. 6. **MALDI-MS spectra of the reducing end sequencing analysis of fraction D.** *Panel A* shows the mass spectrum of the analysis of the intact octasaccharide after derivatization. The observed mass of 2008.3 is consistent with a single label attached to the reducing end. *Panel B* shows the MALDI-MS spectrum of the end-labeled tetrasaccharide arising from heparin lyase II digestion of the semicarbazide-labeled octasaccharide. *Panel C* shows the chemical reactions of the reducing end sequencing analysis. *P* represents peptide $(Arg\text{-}Gly)_{19}$-Arg; *Sac* + *P* represents the complex of peptide and octasaccharide.

acid residue at the nonreducing end, was subjected to exolytic enzyme digestions. We found that the tetrasaccharide was resistant to the digestions by iduronate-2-sulfatase, α-iduronidase, and β-glucuronidase (data not shown). The result suggests that residue 4 is a 2-*O*-sulfated glucuronic acid as it is known to be unsusceptible to digestions by any of these enzymes. It is known that glucuronate-2-sulfatase specifically removes the sulfate from 2-*O*-sulfated glucuronic acid residue (36). However, because of the unavailability of glucuronate-2-sulfatase, we could not determine whether the resultant tetrasaccharide was susceptible to digestion. We also found that fraction D was resistant to heparin lyase II mutant (C348A) digestion (data not shown). The mutated enzyme cleaves glycosidic linkages containing unsulfated uronic acids but not those containing sulfated iduronic acids (35) and sulfated glucuronic acids.[9] The latter result hinted that residue 4 is a 2-*O*-sulfate glucuronic acid residue. However, we also found that an octasaccharide, containing the linkage of

[9] Z. Shriver and R. Sasisekharan, unpublished observation.

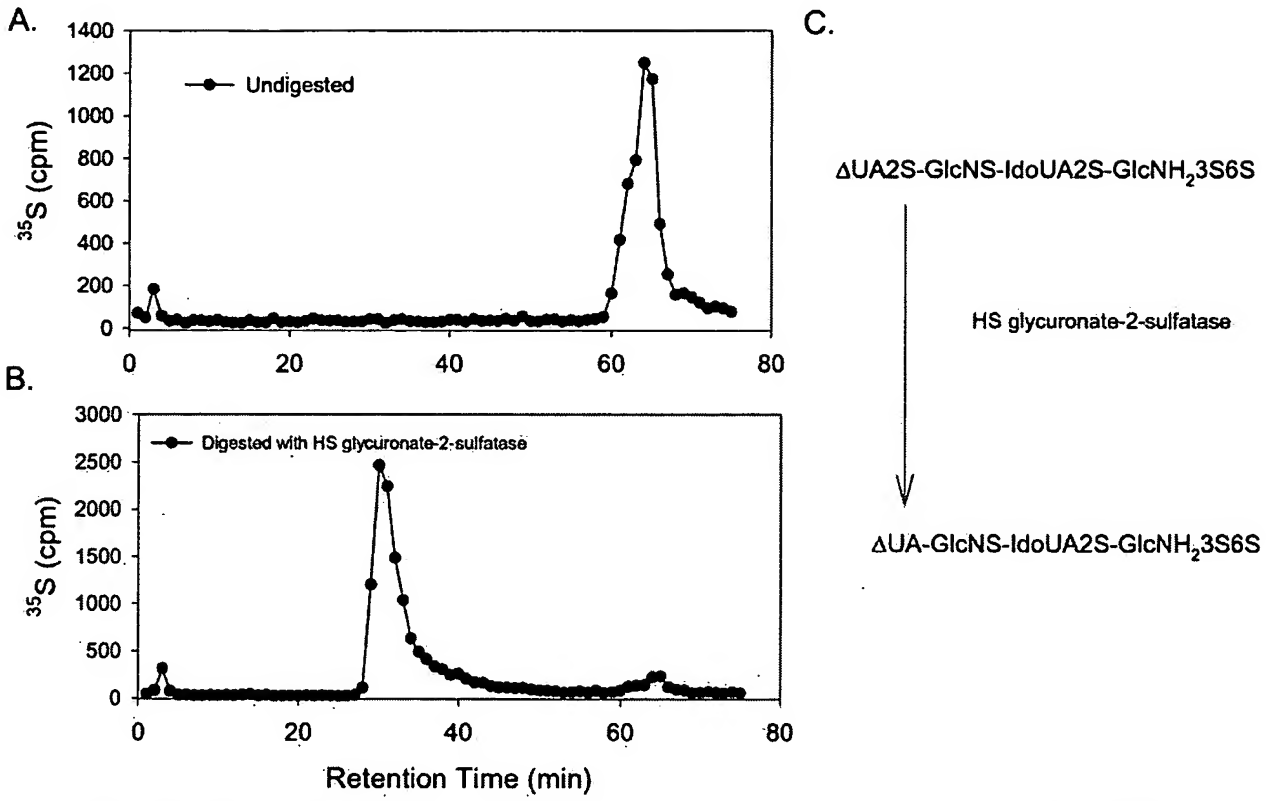


FIG. 7. **DEAE-NPR-HPLC chromatograms of the reducing end tetrasaccharide (residues 1–4) before and after HS glycuronate-2-sulfatase digestion.** The reducing tetrasaccharide was obtained by subjecting fraction D to heparin lyase II digestion. The resultant tetrasaccharide was then digested with HS glycuronate-2-sulfatase. *Panel A* shows the chromatogram of the undigested tetrasaccharide. *Panel B* shows the chromatogram of glycuronate-2-sulfatase-digested tetrasaccharide. *Panel C* depicts action of the HS glycuronate-2-sulfatase digestion.

–GlcNAc-IdoUA2S-, was resistant to digestion by the heparin lyase II mutant (C348A).[10]

DISCUSSION

HS is a common receptor for numerous viruses. It is believed that the defined sulfated sequences determine the specificity for herpes simplex virus (13, 19, 20). Because of the structural complexity of HS, the structural specificity of HS-based receptors is still unknown. It is apparent that understanding the relationship between the saccharide sequences and their activities in promoting viral infection will permit us to delineate HS-assisted viral infections at the molecular level. A previous study has shown that the interaction of 3-*O*-sulfated HS, generated by 3-OST-3 but not by 3-OST-1, interacts with gD to induce the entry of HSV-1 into target cells (13). In the present study, a gD-binding octasaccharide was prepared and purified from a HS oligosaccharide library that was modified by 3-OST-3. The sequence of the isolated gD-binding site is ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S. To our knowledge, this structure has not been previously reported in HS or heparin. As expected, a 3-*O*-sulfated *N*-unsubstituted glucosamine residue was found (residue 1). The result is consistent with the previously characterized substrate specificity of 3-OST-3A (11).

The binding affinity between gD and the purified octasaccharide was determined to be 18 μM. We noted that the gD-binding affinity of the octasaccharide is about 10-fold lower than that of the intact 3-OST-3-modified HS. Two possibilities may contribute to the lower binding affinity. First, two domains in gD for binding to 3-*O*-sulfated HS were predicted by Carfi and colleagues (21). It is possible that the HS polysaccharide interacts with both sites, whereas the octasaccharide is insufficiently large to bind at both sites. Second, both α- and β-anomeric isomers of octasaccharides are likely generated by heparin lyase III depolymerization. The binding affinities of the α- and β-anomeric isomers of the octasaccharide may be different. At the polysaccharide level, the glucosamine residue is present in only the α-form.

The results from the sequencing analysis of the octasaccharide suggested that residue 4 is a 2-*O*-sulfated uronic acid. Two lines of evidence hinted that residue 4 is a 2-*O*-sulfated glucuronic acid residue. First, our results showed that residue 4 is resistant to digestion by β-glucuronidase, α-iduronidase, or iduronate-2-sulfatase. We noted that 2-*O*-sulfated glucuronic acid is resistant to digestions by these enzymes. Second, the octasaccharide is susceptible to the wild type heparin lyase II digestion, but not susceptible to digestion by a heparin lyase II mutant (C348A). Heparin lyase II cleaves the linkages that contain 2-*O*-sulfated glucuronic acid residue, whereas the mutated enzyme, heparin lyase II (C348A), does not cleave this type of linkage.[11] In addition, a previous report demonstrated

[10] In addition to the results presented in a prior publication (35), we tested the susceptibilities of three oligosaccharides to the digestion by heparin lyase II mutant (C348A). Two of them were tetrasaccharides with structures of ΔUA2S-GlcNS6S-GlcUA2S-GlcNS and ΔUA2S-GlcNS6S-GlcUA2S-GlcNS6S (43). One is an octasaccharide with a structure of ΔUA2S-GlcNAc-IdoUA2S-GlcNAc-IdoUA2S-GlcNAc-IdoUA2S-GlcNAc (38). All three of the oligosaccharides were resistant to the digestion.

[11] We failed to detect any ^{3}H-labeled monosulfated disaccharides from the high pH (4.5) and low pH (1.5) nitrous acid-degraded fraction D (10 pmol) followed by [^{3}H]sodium borohydride (5–15 Ci/mmol) reduction despite the fact that IdoUA2S-[^{3}H]AnMan3S6S was detected. It is mainly because of ^{3}H-labeled contaminants in the sodium [^{3}H]borohydride. Those contaminants were eluted near the region where ^{3}H-labeled monosulfated disaccharides were eluted on RPIP-HPLC.

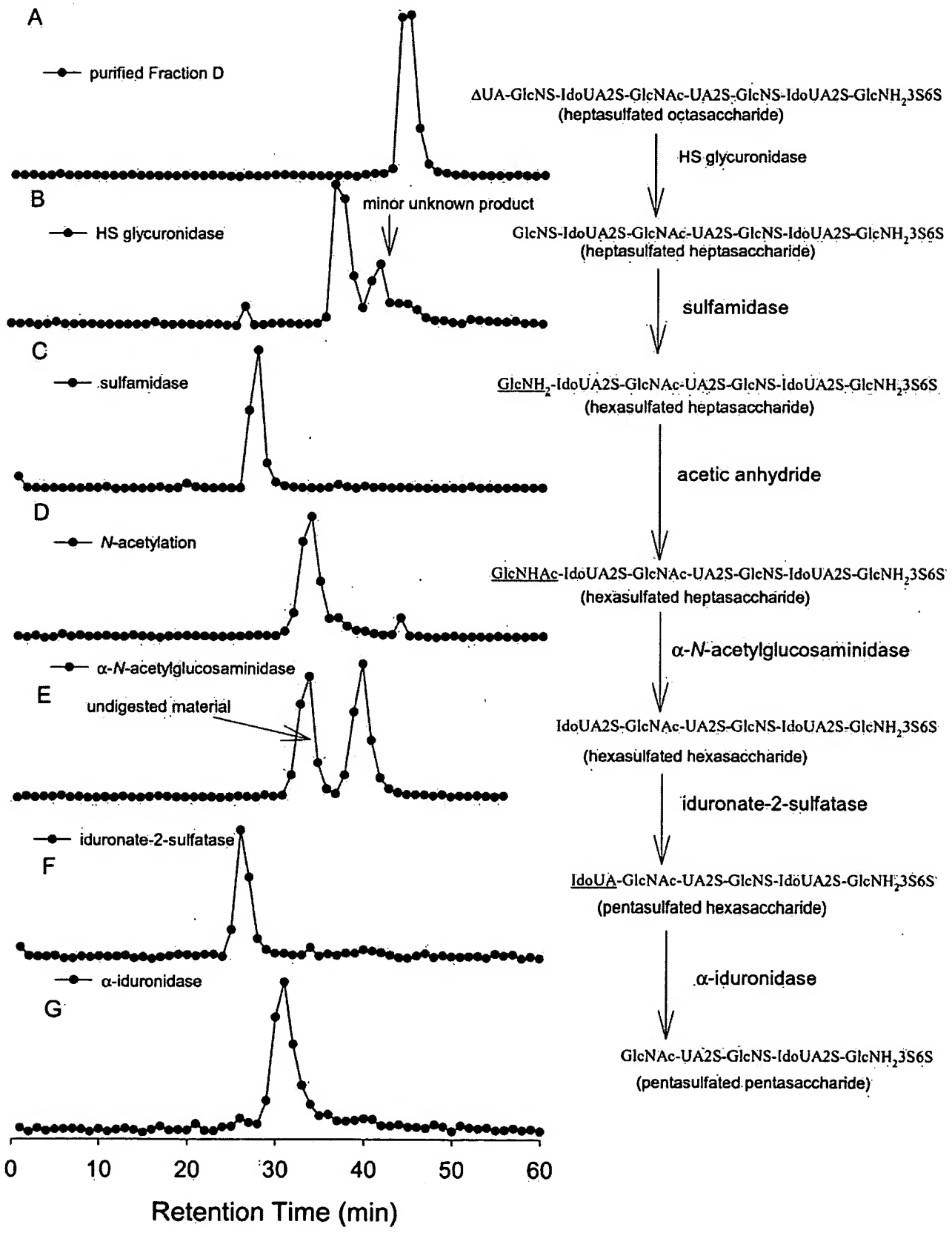


FIG. 8. **Nonreducing end sequencing analysis of fraction D.** *Panels A–G* shows the profiles of enzymatically digested fraction D on DEAE-NPR-HPLC. Between the steps of the sequencing analysis, the designated product was purified by DEAE-NPR-HPLC followed by desalting. The action of the enzymatic digestions at each sequencing step is also indicated. The "minor unknown product" (*panel B*) was removed by HPLC before sulfaminidase digestion. Likewise, the "undigested material" (*panel E*) was removed HPLC before iduronate-2-sulfatase digestion.

that the 2-*O*-sulfated glucuronic acid is present in the HS that is isolated from bovine kidney (37). More convincing evidence is required to conclude with certainty that residue 4 is a 2-*O*-sulfated glucuronic acid. Whereas high resolution NMR spectroscopy could solve this question, it requires significantly larger quantities of octasaccharide than is currently available (38).

It is also very important to note that the disaccharide sequence of –GlcNAc-IdoUA2S– was specifically excluded from the HS or heparin from mammalian cells (39). This conclusion is based on the substrate specificity of HS epimerase that converts glucuronic acid to iduronic acid (40). It is known that an *N*-sulfated glucosamine residue that is linked to a glucuronic acid at the nonreducing end is "absolutely" required for the action of epimerase (39). Thus, the sequences of –GlcNS-IdoUA– and –GlcNS-IdoUA2S– are present in HS, whereas, the sequences of –GlcNAc-IdoUA- and –GlcNAc-IdoUA2S– are not. It should be noted that the sequence of –GlcNAc-IdoUA2S– was isolated from *Achatina fulica*, suggesting that the epimerase may have different substrate specificities from different organisms (38).

We also noted that –GlcNAc-GlcUA2S– has not been discovered in HS that is isolated from natural sources. The 2-*O*-sulfated glucuronic acid residue is a rare constituent of HS, and was found in HS isolated from the adult human cerebral cortex, a nuclear fraction from hepatocytes, HS from bovine kidney, and heparin from porcine intestines (41–43). This residue is synthesized by HS 2-*O*-sulfotransferase, although the enzyme preferably generates 2-*O*-sulfated iduronic acid (IdoUA2S) (44). To this end, it is still not known whether the presence of a 2-*O*-sulfated glucuronic acid, or possibly a 2-*O*-sulfated iduronic acid, residue is essential for gD binding. A comprehensive study of the relationship of the saccharide sequences and gD-binding affinity remains to be investigated when a series of structurally defined HS oligosaccharides are available. The structural information from this study will serve as a lead compound for the chemical synthesis of HS oligosaccharides for further investigation (45).

It is now widely accepted that HS contains both high and low sulfated domains (46). The highly sulfated domains, containing the repeating trisulfated disaccharides of –IdoUA2S-GlcNS6S–, has been the focus of a number of studies investigating HS-related biological functions. For example, the highly sulfated domains bind to fibroblast growth factors and fibroblast growth factor receptors to exhibit various biological functions. Furthermore, previous reports suggest that the antithrombin-binding site is also located within highly sulfated domains (27, 47). It is interesting to note that the gD-binding octasaccharide contains two motifs with distinct sulfation levels. The low sulfated domain, residues 5–8, is composed of one sulfate group per disaccharide, and the highly sulfated domain, residues 1–4, is composed of an average of 2.5 sulfate groups per disaccharide. Thus, these results suggest that the gD-binding site contains both a highly sulfated domain and a low sulfated domain. The crystal structure of gD and HveA, a previously characterized herpes simplex viral entry receptor, predicts two potential 3-*O*-sulfated HS-binding sites (21). One binding site is located in a deep surface pocket with only three basic amino acid residues. This proposed binding site is very close to the binding site of gD and HveA, suggesting that this site might be involved in functional changes in gD. Their observation suggests that the binding of 3-*O*-sulfated HS to this site might involve a small number of positive amino acid residues (from gD) and small number of negatively charged sulfate groups (from HS). A second 3-*O*-sulfated HS-binding site was identified on a relatively flat surface with numerous basic amino acid residues. The second site is away from the HveA-binding site on gD. Carfi and colleagues (21) suggest that the second site in gD might provide the ionic interaction sites to bind to 3-*O*-sulfated HS. It remains to be investigated at which binding sites in gD the isolated octasaccharide interacts.

In summary, an approach to isolate and characterize an octasaccharide that binds to gD was described. It is still not known if this octasaccharide is the minimum necessary sequence for assisting HSV-1 entry into cells. Nevertheless, because the interaction of gD and 3-*O*-sulfated HS is a key step for triggering the fusion of virus and cells, our study provides valuable structural information for determining the specific roles of HS in assisting HSV-1 infections, as well as in the development of therapeutic agents for treating HSV-1 infection.

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REFERENCES

1. Liu, J., and Thorp, S. C. (2002) *Med. Res. Rev.* **22,** 1–25
2. Rosenberg, R. D., Showrak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) *J. Clin. Invest.* **99,** 2062–2070
3. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu. Rev. Biochem.* **68,** 729–777
4. Alexander, C. M., Reichsman, F., Hinkes, M. T., Lincecum, J., Becker, K. A., Cumberledge, S., and Bernfield, M. (2000) *Nat. Genet.* **25,** 329–332
5. Reizes, O., Lincecum, J., Wang, Z., Goldberger, O., Huang, L., Kaksonen, M., Ahima, R., Hinkes, M. T., Barsh, G. S., Rauvala, H., and Bernfield, M. (2001) *Cell* **106,** 105–116
6. Lindahl, U., Kusche-Gullberg, M., and Kjellen, L. (1998) *J. Biol. Chem.* **273,** 24979–24982
7. Esko, J. D., and Lindahl, U. (2001) *J. Clin. Invest.* **108,** 169–173
8. Liu, J., Shworak, N. W., Sinaÿ, P., Schwartz, J. J., Zhang, L., Fritze, L. M. S., and Rosenberg, R. D. (1999) *J. Biol. Chem.* **274,** 5185–5192
9. Aikawa, J.-i., Grobe, K., Tsujimoto, M., and Esko, J. D. (2001) *J. Biol. Chem.* **276,** 5876–5882
10. Habuchi, H., Tanaka, M., Habuchi, O., Yoshida, K., Suzuki, H., Ban, K., and Kimata, K. (2000) *J. Biol. Chem.* **275,** 2859–2868
11. Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., and Rosenberg, R. D. (1999) *J. Biol. Chem.* **274,** 38155–38162
12. Liu, J., Shworak, N. W., Fritze, L. M. S., Edelberg, J. M., and Rosenberg, R. D. (1996) *J. Biol. Chem.* **271,** 27072–27082
13. Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) *Cell* **99,** 13–22
14. Spear, P. G., Eisenberg, R. J., and Cohen, G. H. (2000) *Virology* **275,** 1–8
15. Shukla, D., and Spear, P. G. (2001) *J. Clin. Invest.* **108,** 503–510
16. Mettenleiter, T. C. (1989) *Virology* **171,** 623–625
17. Herold, B. C., WuDunn, D., Soltys, N., and Spear, P. G. (1991) *J. Virol.* **65,** 1090–1098
18. Trybala, E., Bergstrom, T., Svennerholm, B., Jeansson, S., Glorioso, J. C., and Olofsson, S. (1994) *J. Gen. Virol.* **75,** 743–752
19. Feyzi, E., Trybala, E., Bergstrom, T., Lindahl, U., and Spillmann, D. (1997) *J. Biol. Chem.* **272,** 24850–24857
20. Herold, B., Gerber, S. I., Belval, B. J., Siston, A. M., and Shulman, N. (1996) *J. Virol.* **70,** 3461–3469
21. Carfi, A., Willis, S. H., Whitbeck, J. C., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Wiley, D. C. (2001) *Mol. Cell* **8,** 169–179
22. Hernaiz, M., Liu, J., Rosenberg, R. D., and Linhardt, R. J. (2000) *Biochem. Biophys. Res. Commun.* **276,** 292–297
23. Godavarti, R., and Sasisekharan, R. (1998) *J. Biol. Chem.* **273,** 248–255
24. McLean, M. W., Bruce, J. S., Long, W. F., and Williamson, F. B. (1984) *Eur. J. Biochem.* **145,** 607–615
25. Nicola, A. V., Willis, S. H., Naidoo, N. N., Eisenberg, R. J., and Cohen, G. H. (1996) *J. Virol.* **70,** 3815–3822
26. Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P., and Gallagher, J. T. (1998) *J. Biol. Chem.* **273,** 22936–22942
27. Zhang, L., Yoshida, K., Liu, J., and Rosenberg, R. D. (1999) *J. Biol. Chem.* **274,** 5681–5691
28. Shriver, Z., Raman, R., Venkataraman, G., Drummond, K., Turnbull, J., Toida, T., Linhardt, R., Biemann, K., and Sasisekharan, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10359–10364
29. Pope, M., Raska, C., Thorp, S. C., and Liu, J. (2001) *Glycobiology* **11,** 505–513
30. Rhomberg, A. J., Ernst, S., Sasisekharan, R., and Biemann, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4176–4181
31. Lee, M. K., and Lander, A. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2768–2772
32. Venkataraman, G., Shriver, Z., Raman, R., and Sasisekharan, R. (1999) *Science* **286,** 537–542
33. Yamada, S., Yoshida, K., Sugiura, M., Sugahara, K., Khoo, K. H., Morris,

H. R., and Dell, A. (1993) *J. Biol. Chem.* **268**, 4780–4787
34. Warnick, C. T., and Linker, A. (1972) *Biochemistry* **11**, 568–572
35. Rhomberg, A. J., Shriver, Z., Biemann, K., and Sasisekharan, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12232–12237
36. Freeman, C., and Hopwood, J. (1989) *Biochem. J.* **259**, 209–216
37. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem.* **271**, 17804–17810
38. Kim, Y. S., Ahn, M. Y., Wu, S. J., Kim, D., Toida, T., Teesch, L. M., Park, Y., Yu, G., Lin, J., and Linhardt, R. J. (1998) *Glycobiology* **8**, 869–877
39. Conrad, H. E. (1998) *Heparin-binding Proteins*, pp. 7–60, Academic Press, San Diego, CA
40. Li, J.-P., Gong, F., Darwish, K. E., Jalkanen, M., and Lindahl, U. (2001) *J. Biol. Chem.* **276**, 20069–20077
41. Lindahl, B., Eriksson, L., and Lindahl, U. (1995) *Biochem. J.* **306**, 177–184
42. Fedarko, N. S., and Conrad, H. E. (1986) *J. Cell Biol.* **102**, 587–599
43. Yamada, S., Murakami, T., Tsuda, H., Yoshida, K., and Sugahara, K. (1995) *J. Biol. Chem.* **270**, 8696–8705
44. Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2001) *Biochemistry* **40**, 5548–5555
45. Petitou, M., Herault, L.-P., Bernat, A., Driguez, P.-A., Duchaussoy, P., Lormeau, J.-C., and Herbert, J.-M. (1999) *Nature* **398**, 417–422
46. Gallagher, J. T. (2001) *J. Clin. Invest.* **108**, 357–361
47. Jin, L., Abrahams, P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) *Proc. Natl. Acad. Sci.* **94**, 14683–14688

Portable sulphotransferase domain determines sequence specificity of heparan sulphate 3-O-sulphotransferases

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3-*O*-Sulphates are the rarest substituent of heparan sulphate and are therefore ideally suited to the selective regulation of biological activities. Individual isoforms of heparan sulphate D-glucosaminyl 3-O-sulphotransferase (3-OST) exhibit sequence-specific action, which creates heparan sulphate structures with distinct biological functions. For example, 3-OST-1 preferentially generates binding sites for anti-thrombin, whereas 3-OST-3 isoforms create binding sites for the gD envelope protein of herpes simplex virus 1 (HSV-1), which enables viral entry. 3-OST enzymes comprise a presumptive sulphotransferase domain and a divergent N-terminal region. To localize determinants of sequence specificity, we conducted domain swaps between cDNA species. The N-terminal region of 3-OST-1 was fused with the sulphotransferase domain of 3-OST-3_A to generate N1-$ST3_A$. Similarly, the N-terminal region of 3-OST-3_A was fused to the sulphotransferase domain of 3-OST-1 to generate $N3_A$-ST1. Wild-type and chimaeric enzymes were transiently expressed in COS-7 cells and extracts were analysed for selective generation of binding sites for anti-thrombin. 3-OST-1 was 270-fold more efficient at forming anti-thrombin-binding sites than 3-OST-3_A, indicating its significantly greater selectivity for substrates that can be 3-O-sulphated to yield such sites. $N3_A$-ST1 was as active as 3-OST-1, whereas the activity of N1-$ST3_A$ was as low as that of 3-OST-3_A. Analysis of Chinese hamster ovary cell transfectants revealed that only 3-OST-3_A and N1-$ST3_A$ generated gD-binding sites and conveyed susceptibility to infection by HSV-1. Thus sequence-specific properties of 3-OSTs are defined by a self-contained sulphotransferase domain and are not directly influenced by the divergent N-terminal region.

Key words: anti-thrombin, anti-coagulant, gD viral glycoprotein, herpes simplex virus.

INTRODUCTION

Higher organisms express heparan sulphate (HS) proteoglycans on cell surfaces and within extracellular matrixes. HS consists of a repeated disaccharide unit, GlcNAcα1/4HexAβ1–4 (in which HexA represents L-iduronic or D-glucuronic acid), which contains *N*-acetyl, *N*-sulphate, N-unsubstituted and O-sulphate groups. In large part, the specific arrangement of sulphate moieties gives rise to distinct binding sequences that activate ligands, such as anti-thrombin (AT) and growth factors, and thereby regulate numerous biological processes including anti-coagulation, angiogenesis and susceptibility to pathogens (reviewed in [1–5]).

Modification of the co-polymer backbone is thought to occur primarily through an ordered series of reactions. Initially subsets of GlcNAc residues are converted to *N*-sulpho-D-glucosamine (GlcNS) by isoforms of HS N-deacetylase/N-sulphotransferase (NDST) [3,6–9]. The four NDST isoforms show distinct enzymic properties that, in part, might govern the final density of N-sulphation and production of rare N-unsubstituted GlcN residues [9–11]. Regions with a high density of GlcNS are then preferentially modified. A single isoform of HS C_5 epimerase converts occasional GlcA residues to IdoA [12,13]. The HS 2-sulphotransferase modifies IdoA residues at high frequency and GlcA residues at low frequency to produce L-iduronic acid 2-*O*-sulphate (IdoA2S) or 2-O-sulphated glucuronic acid (GlcA2S) respectively [14,15]. HS 6-O-sulphotransferase next converts occasional glucosamine residues to 6-O-sulphated glucosamine [16]. Three isoforms are known with distinct specificities that are dependent, in part, on the specific epimer of an adjacent uronic acid [4]. Finally, the rarest HS modification, 3-O-sulphation of glucosamine, is added by the sequence-specific action of HS D-glucosaminyl 3-O-sulphotransferase (3-OST). There are at least five different isoforms, each of which is expressed in a distinctive pattern of cells and tissues [5]. Given these properties, the 3-OST multigene family is exquisitely suited to regulate HS biological diversity through the production of distinct fine structures.

For example, the isoform 3-OST-1 is rate-limiting for endothelial cell production of anti-coagulant HS (HS^{act}) [17–20]. The enzyme preferentially modifies a specific precursor sequence to create the AT-binding site -Glc(NS/Ac)6S-GlcA-GlcNS3S±6S-IdoA2S-GlcNS6S- [18,21,22] (in which GlcNS3S±6S represents *N*-sulpho-D-glucosamine 3-*O*-sulphate or *N*-sulpho-D-glucosamine 3,6-*O*-bisulphate). On binding this sequence, a conformational change is induced in AT that markedly enhances the rate at which AT neutralizes coagulation proteases. The central 3-*O*-sulphate (shown in bold type) is absolutely essential for both high-affinity binding and activation of AT [21,23,24].

In contrast, the 3-OST-3 isoforms exhibit a distinct biology owing to a very different specificity [25,26]. Modification of HS by 3-OST-3_A, creates a heparin lyase-resistant tetrasaccharide with the novel sequence ΔUA2S-GlcNS-IdoA2S-GlcN3S±6S

Abbreviations used: 3-OST, heparan sulphate D-glucosaminyl 3-O-sulphotransferase; AT, anti-thrombin; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GlcNS, *N*-sulpho-D-glucosamine; HS, heparan sulphate; HS^{act}, HS with AT-binding sites; HSV-1, herpes simplex virus 1; IdoA2S, L-iduronic acid 2-O-sulphate; NDST, HS N-deacetylase/N-sulphotransferase; PAPS, adenosine 3′-phosphate 5′-phosphosulphate.

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(in which ΔUA represents $\Delta^{4,5}$-unsaturated uronic acid) [25]. This structure might contribute to infection by herpes simplex virus type 1 (HSV-1). HSV-1 initially binds to cell-surface HS by interactions of the envelope glycoproteins gC and/or gB (reviewed in [27]). The interaction of an additional viral glycoprotein, gD, with one of several receptors then leads to viral entry through fusion of the virion envelope to the cell membrane (reviewed in [28]). Shukla et al. [29] have demonstrated that modification by 3-OST-3 enzymes selectively creates HS sites for binding gD and for initiating HSV-1 entry.

The determinants of 3-OST specificity are unknown. All 3-OST enzymes comprise a divergent N-terminal domain and a homologous C-terminal domain. The conserved region shows similarity to most sulphotransferases and thereby might constitute a functional sulphotransferase domain [5,19,30,31]. This assertion is supported by the crystal structure of the very similar and homologous sulphotransferase domain from NDST1 [32]. However, the N-terminal domain might additionally contribute to sulphotransferase specificity. The 3-OST divergent domain lacks similarity in primary structure but does show a conserved regional organization, with most enzymes having a type II integral membrane architecture [5]. The lone exception is 3-OST-1, which is an intraluminal resident protein [19]. Despite this difference, the divergent domain is always extremely enriched in Ser, Pro, Leu, Ala and Gly residues. Conservation of composition suggests an important functional role. This divergent region might conceivably directly influence isoform sequence specificity.

In the present work we investigate whether sequence-specific modification is directly dependent on the N-terminal region by constructing proteins chimaeric between the functionally distinct 3-OST-1 and 3-OST-3_A. The results indicate the 3-OST conserved region is a self-contained sulphotransferase domain encompassing determinants for both catalysis and sequence specificity.

EXPERIMENTAL

Plasmids and recombinant proteins

pcDNA3.1-based expression plasmids containing mouse 3-OST-1 (pCMV-3-OST) or human 3-OST-3_A (pcDNA3-JL3.3) were described previously [5,19]. Recombinant mouse 3-OST-1 and human 3-OST-3_A were purified from baculovirus expression systems [25,33] and were gifts from Dr Jian Liu (University of North Carolina, Chapel Hill, NC, U.S.A.). gD-1:Fc, a fusion of the gD ectodomain and the Fc of rabbit IgG, was purified from PEAK® (Edge Biosystems) cell transfectants, as described [29].

Construction of expression plasmids

pN3_A-ST1 was constructed by three-piece ligation. A 203 bp fragment, the distal N-terminal domain of 3-OST-3_A with 9 nt of 3-OST-1 and an *Ear*I site, was PCR generated from pcDNA3-JL3.3 with 5′-dGGCGGCACAGAGAAAGCGCCTCCTGCAACT-3′ and 5′-dCTCTTCGTGTGGAACCTTCGTCCAGGAGCAGCGCCAGG-3′ primers; the product was cloned into PCR-Script Cam (Stratagene). The insert was released as a *Kas*I/*Kpn*I fragment, then partial *Ear*I digestion generated fragment 1, a 157-bp *Kas*I/*Ear*I fragment. Next a 1.4 kb fragment containing an *Ear*I site preceding the sulphotransferase domain of mouse 3-OST-1 was generated from pCMV-3-OST by PCR with 5′-dGCCTCAGAAGCCATAGAGCCCACCGCAT-3′ and 5′-dCTCTTCCACACAGCAGCTGCCACAGACCATCATCATTGG-3′ primers; the product was cloned into PCR-Script Cam. Fragment 2 was then released as a 1.1 kb *Ear*I/*Xba*I fragment. Fragments 1 and 2 were cloned into *Kas*I/*Xba*I-cleaved pcDNA3-JL3.3 to generate pN3_A-ST1. pN1-ST3_A formation required silent mutations to introduce *Bam*HI sites at the domain border. First a 1.1 kb fragment *Hin*dIII fragment from pCMV-3-OST was subcloned into pUC18 (GenBank® accession number L09136) to create a mutation template. A *Bam*HI site was introduced with a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and two mutagenic primers, 5′-dCAGCATCCAATGGATCCACACAGCAG-3′ and 5′-dCTGCTGTGTGGATCCATTGGATGCTG-3′ (*Bam*HI site underlined). Digestion with *Bam*HI produced a 524 bp fragment, N1, containing the N-terminal domain of 3-OST-1. Next a 2.8 kb *Sma*I fragment was isolated from pcDNA3-JL3.3 and amplified by PCR with 5′-dCCTGGACGAAGGATCCAAGCAGCTGCC-3′ (*Bam*HI site underlined) and 5′-dGCGTCTTCTCCATGGTGATCTG-3′ primers; the 219 bp product was cloned into PCR-Script Cam. Digestion with *Eco*RI and *Kpn*I released a 216 bp fragment that was cloned into an *Eco*RI/partly *Kpn*I-cleaved pcDNA3-JL3.3 plasmid to reconstitute the 3-OST-3_A sulphotransferase domain. Finally, this last plasmid was digested with *Bam*HI and the above N1 fragment was inserted to create pN1-ST3_A. The reading frame and entire coding region of all constructs were confirmed by sequencing.

Transient expression in COS-7 cells

COS-7 cells (A.T.C.C., CRL-1651) were grown in Dulbecco's modified Eagle's medium (Life Technologies) containing 10% (v/v) fetal bovine serum (FBS) (Life Technologies) with 100 μg/ml streptomycin and 100 i.u./ml penicillin at 37 °C under a humidified air/CO_2 (19:1) atmosphere. For each construct a six-well plate (Falcon) was seeded with 7.5×10^4 cells and grown for 2 days (60–90% confluence). Monolayers were washed with PBS, then 6 ml of serum-free medium containing 12 μg of construct DNA and 60 μl of GenePORTER (Gene Therapy Systems) was split between six wells. After growth for 3–5 h, 1 vol. of medium with 20% (v/v) FBS was added. After growth for 24 h, monolayers were washed with PBS and incubated with fresh medium for 36 h. Cells from a single plate were harvested by treatment with trypsin and washed with PBS; the pellet was snap-frozen in liquid nitrogen and stored at −80 °C. Extracts were generated by vortex-mixing pellets in 100 μl of ice-cold 0.25 M sucrose with 1% (v/v) Triton X-100, then removing the insoluble residue by centrifugation at 10000 *g* for 10 min. For analysis of secreted sulphotransferase activity, the final growth incubation was conducted in a previously described serum-free medium [20], which was clarified by centrifugation and then frozen as above.

Preparation of [^{35}S]HS

Chinese hamster ovary (CHO)-K1 cells (a gift from Dr Lijuan Zhang, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.) were inoculated at 38 000 cells/cm² in a 75 cm² flask, then grown at 37 °C under air/CO_2 (19:1) in Ham's F-12 medium (Life Technologies) with 10% (v/v) FBS. At 2 days after inoculation, the monolayer was washed with PBS, then incubated for 16 h in 4 ml of Ham's F-12 medium with 50 mCi of carrier-free $Na_2{}^{35}SO_4$ (ICN) and 10% (v/v) dialysed FBS. Cell-surface [^{35}S]HS was then purified as described previously [18].

Formation of AT-binding sites (HS^{act} conversion assay)

To ensure that reaction conditions were suitable for both enzymes, we initially evaluated extracts from 3-OST-1, 3-OST-3_A and pcDNA3 transfectants and varied previous conditions

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[18,20,34] to maximize the HS sulphotransferase activity of 3-OST compared with empty-vector controls. The resulting optimal conditions were applied to both the HS^{act} conversion assay and the detection of the HS sulphotransferase activity. The HS^{act} conversion reactions (50 μl) contained 2 μg of cell extract protein, 5×10^4 c.p.m. of [^{35}S]HS from CHO-K1 cells, 500 μM adenosine 3′-phosphate 5′-phosphosulphate (PAPS; Sigma), 50 mM Mes, pH 7.0, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1.2 mg/ml BSA, 60 mM NaCl, 0.2 mg/ml glycogen and 1% (v/v) Triton X-100. Reactions were incubated at 37 °C for 2 h, then 1.25 μl of 100 μM AT was added per reaction. After incubation for 30 min at room temperature the amount of [^{35}S]HS that bound AT (HS^{act}) was determined by AT-affinity microchromatography [17]. Activity was calibrated against standards of purified recombinant 3-OST-1 [33]. One unit of activity generates a 0.5% increase in HS^{act} after 20 min incubation under standard reaction conditions [20].

Analysis of HS sulphotransferase activity in cell extract

Reactions were as described above except that the sulphate acceptor was 1.2 μg of bovine kidney HS (ICN Biomedicals) and the sulphate donor was [^{35}S]PAPS (30 μM, 10^6 c.p.m.). Reactions were incubated at 37 °C for 2 h, quenched at 100 °C for 10 min and centrifuged at 14000 *g* for 10 min. Supernatant was combined with 1 ml of UPAS [35] and incorporation into [^{35}S]HS was determined by analytic DEAE chromatography [35]. Activity was typically 40% higher for 3-OST transfectant extracts than for the empty-vector control. Activity was similarly determined for 10 μl samples of protein-free medium.

HSV-1 entry and detection of cell-surface gD-1 binding

Determination of CHO transfectant susceptibility to HSV-1 entry has been described previously [29,36]. Detection of gD-1 receptors on transfectants with gD-1:Fc was as described previously [29].

RESULTS

Construction of chimaeric proteins between 3-OST-1 and 3-OST-3_A

We applied a domain-swap strategy (Figure 1) to evaluate whether the distinct sequence specificities of 3-OST-1 and

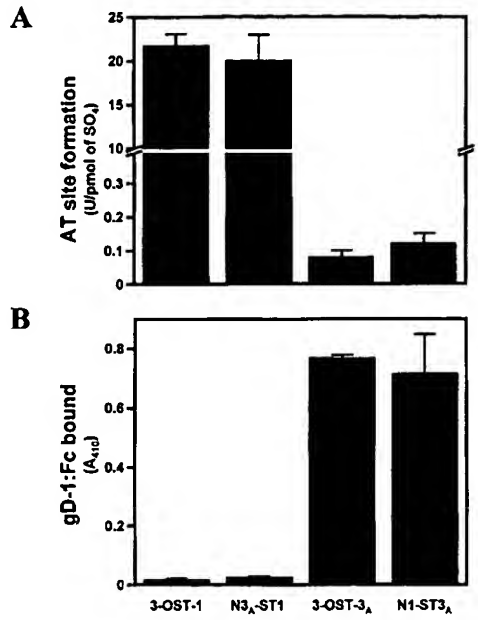

Figure 2 Selective synthesis of ligand-binding sites by 3-OST enzymes

(**A**) Selective synthesis of AT-binding sites was revealed by analysis *in vitro* of cell extracts from COS-7 transfectants. Selectivity was calculated as the HS^{act} conversion activity divided by the net HS sulphotransferase activity. HS^{act} conversion activity was determined by incubating cell extracts (2 μg) with PAPS and [^{35}S]HS lacking 3-*O*-sulphates. The resulting HS forms with nascent AT-binding sites (HS^{act}) were quantified by AT-affinity microchromatography [18]. Extracts from pcDNA3.1 transfectants showed only background conversion activity. Cell extracts (2 μg) were also incubated with [^{35}S]PAPS and HS; incorporated sulphate was then determined by DEAE chromatography and expressed as pmol of sulphate transferred from [^{35}S]PAPS to make [^{35}S]HS. Net HS sulphotransferase activity from 3-OST enzymes was determined as the extract activity minus the value for the empty-vector control. Results are mean ± S.E.M. for six independent transfections. (**B**) Cell surface gD-binding sites synthesized *in vivo* were determined for the indicated CHO transfectants. Monolayers were incubated with gD-1:Fc for 30 min, followed by fixation and incubation with secondary antibody conjugated to horseradish peroxidase. After incubation with substrate the reaction was quenched with 2 M HCl. The enzymic reaction product was detected by measuring A_{410}. Values were corrected for the background binding of gD-1Fc to control pcDNA3 transfectants (A_{410} of 0.393) and are shown as means ± S.D. for three determinations.

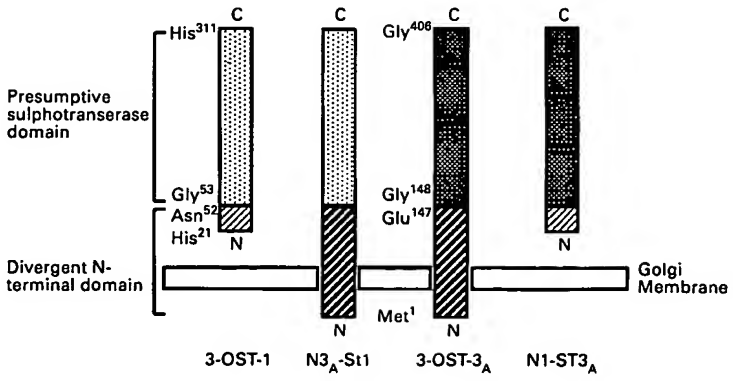

Figure 1 Mature structures of wild-type and chimaeric 3-OST enzymes

N-termini and C-termini are indicated. Met^1 to Glu^{147} of human 3-OST-3_A was fused to Gly^{53} to His^{311} of mouse 3-OST-1 to generate $N3_A$-ST1. Consequently, mature 3-OST-3_A and $N3_A$-ST1 contained a transmembrane domain and were predicted to localize to membranes of the *trans*-Golgi. Similarly, the entire N-terminal region of mouse 3-OST-1 (Met^1 to Asn^{52}) was fused to Gly^{148} to Gly^{406} of human 3-OST-3_A to generate N1-ST3_A. However, mature 3-OST-1 and N1-ST3_A begin at His^{21} owing to signal peptidase cleavage of the 3-OST-1 leader sequence [19]. Thus mature 3-OST-1 and N1-ST3_A are intraluminal resident proteins and localization to the *trans*-Golgi is presumed to involve interaction with a retention protein [19].

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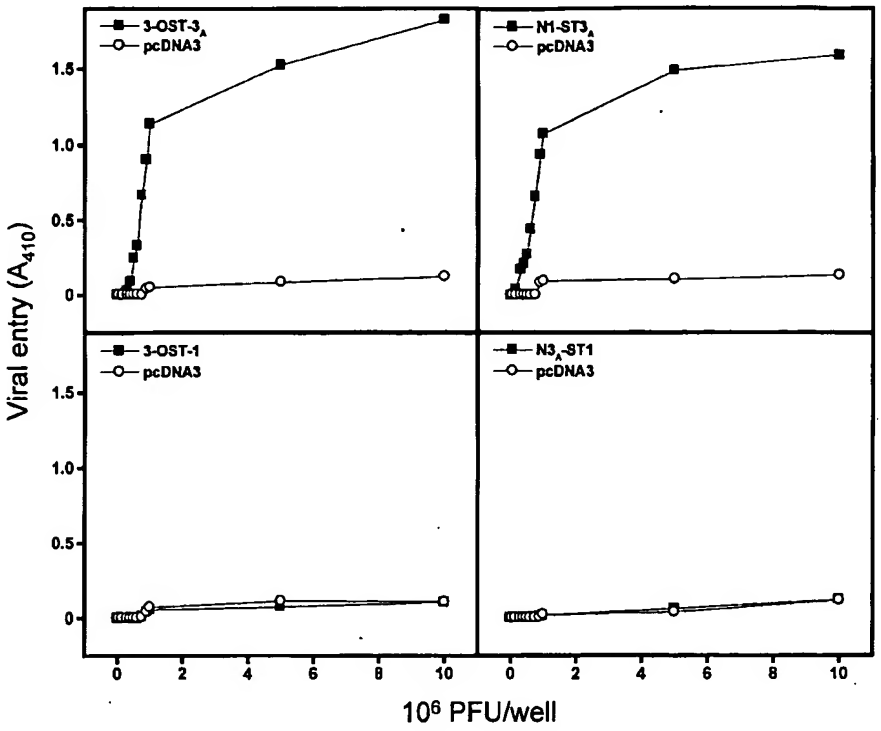

Figure 3 Entry of HSV-1 into CHO cells is mediated only by 3-OST-3_A and N1-$ST3_A$

CHO cells were transfected with the indicated plasmids, then exposed to various concentrations of HSV-1(KOS)gL86, which expresses β-galactosidase from an insert in the viral genome. β-Galactosidase activity, a measure of viral entry, was quantified at 6 h after the addition of virus. Representative values from a single transfection show the amount of reaction product detected spectrophotometrically (A_{410}) as the mean from four wells per virus dose (PFU, plaque-forming units). Standard deviations were typically less than 15% of the mean. Comparable results were obtained in four independent transfection experiments.

3-OST-3_A were determined by their respective divergent N-terminal domains. Comparisons of 3-OST primary structures reveal a tightly demarcated border between the divergent region and the conserved C-terminal domain, which begins with an invariant glycine residue. This conserved span is considered the presumptive sulphotransferase domain of 3-OST enzymes [5]. Thus domain swaps conformed with this clearly defined border. The N-terminal region of 3-OST-1 was fused to the presumptive sulphotransferase domain of 3-OST-3_A to generate N1-$ST3_A$. Similarly, the N-terminal region of 3-OST-3_A was fused to the C-region of 3-OST-1 to generate $N3_A$-ST1. We deliberately avoided the addition of purification tags, which might have altered enzymic function. Each cDNA was subcloned into the pcDNA3.1 expression vector and sequence specificity was evaluated *in vitro* and *in vivo*.

Sequence specificity *in vitro* is unaltered by the divergent N-terminus

We compared the efficiency of wild-type and chimaeric enzymes *in vitro* towards generating AT-binding sites, the preferred product of 3-OST-1. COS-7 cells were transiently transfected with 3-OST expression vectors or the pcDNA3.1 control vector; cell extracts were then evaluated for HS^{act} conversion activity, which measures the 3-OST-dependent formation of AT-binding sites. Detection of even low activities was made possible by employing modified reaction conditions that provided for a 10-fold enhanced sensitivity, compared with earlier assay formats [18,20,34]. Expression levels were inherently variable with transient transfections. To compensate for this, the HS^{act} conversion activity was standardized to the net increase in extract HS sulphotransferase activity (Figure 2A). Transfectional variation was also averaged out by performing several independent transfections. The sensitive assay conditions revealed, for the first time, that the 3-OST-3_A isoform was capable of generating AT-binding sites, albeit at a low activity (0.08 ± 0.02 unit/pmol of SO_4; Figure 2A). This result was validated by analysis of purified recombinant 3-OST-3_A [25], which showed a similar activity (0.14 ± 0.04 unit/pmol of SO_4, $n = 5$). [The low efficiency of purified recombinant 3-OST-3_A in forming AT-binding sites can be compensated for by mass action. Exhaustive modification of [^{35}S]HS with high enzyme levels maximally converts approx. 20% of HS chains to HS^{act}, which bind AT. A similar approx. 20% plateau is also attained by exhaustive modification of HS with purified 3-OST-1 (results not shown). Thus AT site formation by 3-OST-3_A is a detectable activity that modifies the same precursor population as 3-OST-1.] In contrast with 3-OST-3_A, extracts containing 3-OST-1 were 270-fold more active at forming AT-binding sites (Figure 2A). Again, the activity determined for purified recombinant 3-OST-1 enzyme (40 ± 5.7 units/pmol of SO_4, $n = 3$; results not shown) was similar to that obtained from cell extracts (21.80 ± 1.27 units/pmol of SO_4; Figure 2A). Most importantly, the high selectivity of the presumptive sulphotransferase domain of 3-OST-1 was unaltered by the N-terminal swaps (Figure 2A; compare 3OST1 with $N3_A$-ST1). The low selectivity of the 3-OST-3_A presumptive sulpho-

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transferase domain was similarly unchanged (Figure 2; compare 3-OST-3_A with N1-$ST3_A$). Taken together, the above results suggest that enzymic determinants of sequence specificity *in vitro* are embodied within the 3-OST conserved C-terminal region.

Sequence specificity *in vivo* is unaltered by the divergent N-terminus

Wild-type and chimaeric enzymes were also examined by an assay *in vivo* that was dependent on the sequence-specific enzyme action of 3-OST-3 isoforms. Modification of HS by these isoforms creates binding sites for the gD envelope protein and renders CHO cells susceptible to infection by HSV-1 [29]. Thus CHO cells were transfected with 3-OST expression constructs and then exposed to a recombinant form of HSV-1; virus entry was monitored (Figure 3). The HS sulphotransferase activity of transfectant cell extracts was also measured, which confirmed that each 3-OST form was successfully expressed (compared with the empty-vector control, the activity of 3-OST transfectants was elevated by 30–80%). Despite successful expression of all constructs, cells became susceptible to HSV-1 entry only after the expression of constructs with the 3-OST-3_A presumptive sulphotransferase domain (Figure 3). Furthermore, the N-terminal domain swaps did not substantially alter the HSV-1 dose response. The formation of cell-surface gD-binding sites was also evaluated by incubating cells with gD-1:Fc. Figure 2(B) shows that substantial levels of cell-surface gD-binding sites were generated only when transfectants expressed the presumptive sulphotransferase domain of 3-OST-3_A. Thus 3-OST-3 sequence-specific properties stem from the conserved C-terminal region.

Domain swaps alter enzyme secretion

3-OST-1 is the only isoform that is not membrane-bound and can be secreted, presumably because the relevant Golgi retention mechanism, if any, is leaky [5,19,26]. Thus the domain swaps should alter cellular secretion. This possibility was tested by measuring the HS sulphotransferase activity within culture medium conditioned by transiently transfected COS-7 cells. Results were generated from three independent transfections and are expressed as fmol of SO_4 transferred from [^{35}S]PAPS to HS per μl of medium (means$\pm$S.D.). For transfectants expressing type II integral membrane constructs the activity was comparable to background (pcDNA3.1, 17 ± 0.9; 3-OST-3_A, 18 ± 1.1; $N3_A$-ST1, 20 ± 2.7). In contrast, intraluminal resident constructs gave an activity in medium that was significantly greater than that of pcDNA3.1 [3-OST-1, 59 ± 6.6, $P < 0.0004$; N1-$ST3_A$, 26 ± 2, $P < 0.004$ (two-tailed Student's t test)]. Thus the N-terminal domain governs cellular retention.

DISCUSSION

Sulphotransferases (EC 2.8.2.), which make up a large superfamily, transfer a sulphuryl group from the donor PAPS to numerous acceptor molecules [37]. Multiple sequence alignments show that virtually all such enzymes share a 260–290-residue C-terminal presumptive sulphotransferase domain, with at least 25–30% similarity between the most functionally divergent members [5,19,31]. The greatest conservation is found in structures that bind and/or catalyse the obligate cofactor PAPS [31,38,39]. In contrast, specificity for the acceptor substrate must be determined by non-conserved residues. For the oestrogen sulphotransferase, residues defining acceptor specificity lie within the sulphotransferase domain [40]. However, this enzyme has

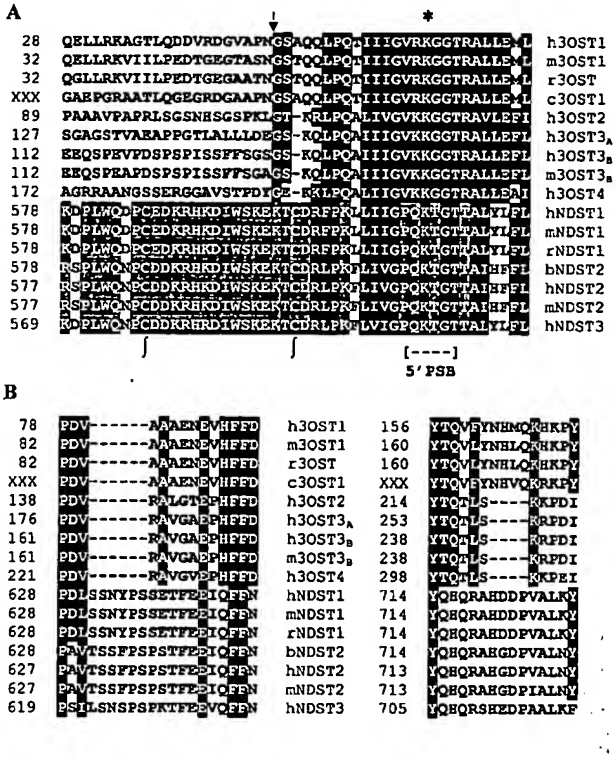

Figure 4 Comparison of sulphotransferase regions

Alignment of amino acid sequences of mammalian 3-OST and NDST isoforms. Consensus residues are indicated for each position where candidates exhibit identical or similar amino acids for all enzymes (black) or only NDST isoforms (grey). Residue numbers are given for each full-length enzyme. (**A**) Junction of 3-OST domains (↓) and the comparable NDST region. Highlighted features include the 5′-phosphosulphate-binding loop (5′PSB) containing the conserved catalytic lysine residue (*). Cysteine residues that formed a disulphide bond are also indicated (∫). (**B**) Two major divergent sulphotransferase regions that might contribute to enzymic sequence specificity. Prefixes indicate the organism of origin: h, *Homo sapiens*; m, *Mus musculus*; r, *Rattus norvegicus*; c, *Canis familiaris*; b, *Bos taurus*. Nucleotide sequences are from the GenBank® database with the following accession numbers: h3OST1, AF019386; m3OST1, AF019385; r3OST, AF177430; c3OST1, AJ388534 (partial sequence); h3OST2, AF105375; h3OST3$_A$, AF105376; h3OST3$_B$, AF105377; m3OST3$_B$, AF168992; h3OST4, AF105378; hNDST1, U36600; mNDST1, AF074926; rNDST1, M92042; bNDST2, AF064825; hNDST2, U36601; mNDST2, P52850; hNDST3, AF074924.

only a single domain [41] and so might not be predictive of the multidomain 3-OSTs. Consequently, we conducted domain swaps to determine whether the divergent domain directly affects the sequence specificity of the presumptive sulphotransferase domain.

Reciprocal swaps were constructed to maintain the clearly defined border between the divergent and conserved domains (Figure 4A, ↓). This site is only 13 residues from the conserved 5′-phosphosulphate-binding loop, which contains an essential catalytic lysine residue (Figure 4A, 5′PSB and asterisk respectively) [5,29,39,42]. Despite such close proximity to critical structures, the chimaeric enzymes were functional. We were able to quantify the sequence selectivity *in vitro* for both 3-OST isoforms by using an improved assay for HS^{act} conversion activity. This approach revealed that 3-OST-3_A was 270-fold less efficient at generating HS^{act} than 3-OST-1. (This value is similar to our earlier estimate of 300-fold. Because 3-OST-3_A activity was initially undetectable, the original estimate was determined by comparing 3-OST-1 activity with the assay detection limit [26]. For 3-OST-1 only approx. 40% of transferred sulphates

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actually create HS^{act} [19,20], suggesting that only approx. 0.15% of 3-OST-3_A-transferred sulphates should occur in AT-binding sites. Although this output seems low, substantial AT-binding sites might be generated in tissues with high enzyme expression.) Most importantly, the enzymic specificity of the sulphotransferase domain *in vitro* was quantitatively unaltered by the swaps. Sequence selectivity was also unaltered *in vivo*. Only constructs with the 3-OST-3_A sulphotransferase domain were capable of forming gD-binding sites and enabling the cellular entry of HSV-1. Combined, the results strongly indicate that the N-terminal domain does not directly alter enzymic substrate specificity. Thus the conserved C-terminal region of 3-OST enzymes is a functionally complete sulphotransferase domain that encodes the determinants for both catalysis and sequence specificity.

Our functional identification of the 3-OST sulphotransferase domain might serve to clarify the border between the two distinct activities of the NDST family. Given the high similarity between these homologous families, the domain margins might correspond tightly [5,19]. It has previously been suggested that the sulphotransferase domain of human NDST1 extends from Leu^{558} to the C-terminus [30,39]. However, X-ray crystallography of this region shows that ordered structure initiates at Asp^{579}. By sequence alignment, this position is 20 residues away from the start of the 3-OST-1 sulphotransferase domain, Gly^{53} (Figure 4A). However, the comparative three-dimensional distance must be much closer, because the NDST span contains a 16-residue cystine-bridged peptide loop (Figure 4A, from ∫ to ∫) [32,43]. Thus Asp^{579} might be the actual start of the NDST sulphotransferase domain. This assertion is in part supported by an NDST1 $Cys^{601} \rightarrow$ Ala mutation, which greatly decreases sulphotransferase activity with minimal effect on deacetylase function [43].

Comparison of 3-OST and NDST primary structures also reveals non-conserved spans that might confer sequence-specific properties [5]. Two such regions are particularly striking (Figure 4B). The structures are not only distinct between 3-OST and NDST families, but differences also occur between 3-OST isoforms. In the crystal structure of human NDST-1 [32], these two non-conserved regions lie on opposite faces of the cleft that binds HS. Moreover, one end of each sequence lies in close proximity to the 5′ phosphosulphate of PAPS. Thus the location of these regions is ideal for restricting active-site access to distinct HS structures. However, full sequence-specific properties of 3-OST isoforms probably involves additional residues because the two regions cover only a portion of the HS-binding groove.

The N-terminal portions of many Golgi enzymes, including NDST1, contain information for appropriate subcellular targeting [44,45]. Although our experiments were not designed to examine subcellular targeting, the domain swaps suggest such a role, given that cellular secretion occurred only for enzymes containing the N-terminal domain of 3-OST-1. We have previously proposed that the divergent region might interact with other HS biosynthetic components and thereby restrict access of selective substrates to the sulphotransferase domain [5]. Thus the N-terminal region might indirectly control sequence specificity. From this perspective it initially seems surprising that the N1-$ST3_A$ construct conveyed HSV-1 entry. Under the conditions used, the N1-$ST3_A$ enzyme clearly had access to appropriate HS precursors to generate a gD-binding site. However, transient transfection results in unphysiologically high expression levels that might flood the Golgi and thereby mask any potential for selective targeting. Thus a more physiologically rigorous approach is necessary to test whether the N-terminal region can indirectly control sequence specificity.

In conclusion, the conserved C-terminal region is a portable and self-contained sulphotransferase domain, which directly determines the specificity of 3-OST isoforms. Having localized the critical region, future studies will be directed towards pinpointing critical residues that define the HS sequence specificity of 3-OST enzymes.

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REFERENCES

1 Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J. and Zhang, L. (1997) Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? J. Clin. Invest. **99**, 2062–2070
2 Lindahl, U., Kusche-Gullberg, M. and Kjellen, L. (1998) Regulated diversity of heparan sulfate. J. Biol. Chem. **273**, 24979–24982
3 Aikawa, J. and Esko, J. D. (1999) Molecular cloning and expression of a third member of the heparan sulfate/heparin GlcNAc *N*-deacetylase/*N*-sulfotransferase family. J. Biol. Chem. **274**, 2690–2695
4 Habuchi, H., Tanaka, M., Habuchi, O., Yoshida, K., Suzuki, H., Ban, K. and Kimata, K. (2000) The occurrence of three isoforms of heparan sulfate 6-*O*-sulfotransferase having different specificities for hexuronic acid adjacent to the targeted *N*-sulfoglucosamine. J. Biol. Chem. **275**, 2859–2868
5 Shworak, N. W., Liu, J., Petros, L. M., Zhang, L., Kobayashi, M., Copeland, N. G., Jenkins, N. A. and Rosenberg, R. D. (1999) Multiple isoforms of heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase. Isolation, characterization, and expression of human cDNAs and identification of distinct genomic loci. J. Biol. Chem. **274**, 5170–5184
6 Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S. J. and Ishihara, M. (1994) Molecular cloning and expression of a glycosaminoglycan *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase from a heparin-producing cell line. J. Biol. Chem. **269**, 2270–2276
7 Hashimoto, Y., Orellana, A., Gil, G. and Hirschberg, C. B. (1992) Molecular cloning and expression of rat liver *N*-heparan sulfate sulfotransferase. J. Biol. Chem. **267**, 15744–15750
8 Eriksson, I., Sandbäck, D., Ek, B., Lindahl, U. and Kjellén, L. (1994) cDNA cloning and sequencing of mouse mastocytoma glucosaminyl N-deacetylase/N-sulfotransferase, an enzyme involved in the biosynthesis of heparin. J. Biol. Chem. **269**, 10438–10443
9 Aikawa, J., Grobe, K., Tsujimoto, M. and Esko, J. D. (2001) Multiple isozymes of heparan sulfate/heparin GlcNAc N-deacetylase/GlcN N-sulfotransferase. Structure and activity of the fourth member, NDST4. J. Biol. Chem. **276**, 5876–5882
10 Cheung, W.-F., Eriksson, I., Kusche-Gullberg, M., Lindahl, U. and Kjellén, L. (1996) Expression of the mouse mastocytoma glucosaminyl *N*-deacetylase/*N*-sulfotransferase in human kidney 293 cells results in increased *N*-sulfation of heparan sulfate. Biochemistry **35**, 5250–5256
11 Pikas, D. S., Eriksson, I. and Kjellen, L. (2000) Overexpression of different isoforms of glucosaminyl N-deacetylase/N-sulfotransferase results in distinct heparan sulfate N-sulfation patterns. Biochemistry **39**, 4552–4558
12 Crawford, B. E., Olson, S. K., Esko, J. D. and Pinhal, M. A. (2001) Cloning, Golgi localization, and enzyme activity of the full-length heparin/heparan sulfate–glucuronic acid c5-epimerase. J. Biol. Chem. **276**, 21538–21543
13 Li, J., Hagner-McWhirter, A., Kjellen, L., Palgi, J., Jalkanen, M. and Lindahl, U. (1997) Biosynthesis of heparin/heparan sulfate. cDNA cloning and expression of D-glucuronyl C5-epimerase from bovine lung. J. Biol. Chem. **272**, 28158–28163
14 Kobayashi, M., Habuchi, H., Yoneda, M., Habuchi, O. and Kimata, K. (1997) Molecular cloning and expression of chinese hamster ovary cell heparan-sulfate 2-sulfotransferase. J. Biol. Chem. **272**, 13980–13985
15 Rong, J., Habuchi, H., Kimata, K., Lindahl, U. and Kusche-Gullberg, M. (2000) Expression of heparan sulphate L-iduronyl 2-O-sulphotransferase in human kidney 293 cells results in increased D-glucuronyl 2-O-sulphation. Biochem. J. **346**, 463–468
16 Habuchi, H., Kobayashi, M. and Kimata, K. (1998) Molecular characterization and expression of heparan-sulfate 6- sulfotransferase. Complete cDNA cloning in human and partial cloning in Chinese hamster ovary cells. J. Biol. Chem. **273**, 9208–9213

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17 Shworak, N. W., Shirakawa, M., Colliec-Jouault, S., Liu, J., Mulligan, R. C., Birinyi, L. K. and Rosenberg, R. D. (1994) Pathway-specific regulation of the synthesis of anticoagulantly active heparan sulfate. J. Biol. Chem. **269**, 24941–24952
18 Shworak, N. W., Fritze, L. M. S., Liu, J., Butler, L. D. and Rosenberg, R. D. (1996) Cell-free synthesis of anticoagulant heparan sulfate reveals a limiting activity which modifies a nonlimiting precursor pool. J. Biol. Chem. **271**, 27063–27071
19 Shworak, N. W., Liu, J., Fritze, L. M. S., Schwartz, J. J., Zhang, L., Logeart, D. and Rosenberg, R. D. (1997) Molecular cloning and expression of mouse and human cDNAs encoding heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase. J. Biol. Chem. **272**, 28008–28019
20 Liu, J., Shworak, N. W., Fritze, L. M. S., Edelberg, J. M. and Rosenberg, R. D. (1996) Purification of heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase. J. Biol. Chem. **271**, 27072–27082
21 Lindahl, U., Bäckström, G., Thunberg, L. and Leder, I. G. (1980) Evidence for a 3-*O*-sulfated D-glucosamine residue in the antithrombin-binding sequence of heparin. Proc. Natl. Acad. Sci. U.S.A. **77**, 6551–6555
22 Rosenberg, R. D. and Lam, L. (1979) Correlation between structure and function of heparin. Proc. Natl. Acad. Sci. U.S.A. **76**, 1218–1222
23 Atha, D. H., Lormeau, J. C., Petitou, M., Rosenberg, R. D. and Choay, J. (1985) Contribution of monosaccharide residues in heparin binding to antithrombin III. Biochemistry **24**, 6723–6729
24 Atha, D. H., Lormeau, J. C., Petitou, M., Rosenberg, R. D. and Choay, J. (1987) Contribution of 3-*O*- and 6-*O*-sulfated glucosamine residues in the heparin-induced conformational change in antithrombin III. Biochemistry **26**, 6454–6461
25 Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R. and Rosenberg, R. D. (1999) Heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase-3A sulfates *N*-unsubstituted glucosamine residues. J. Biol. Chem. **274**, 38155–38162
26 Liu, J., Shworak, N. W., Sinay, P., Schwartz, J. J., Zhang, L., Fritze, L. M. and Rosenberg, R. D. (1999) Expression of heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase isoforms reveals novel substrate specificities. J. Biol. Chem. **274**, 5185–5192
27 Spear, P. G. (1993) Entry of alphaherpes viruses into cells. Semin. Virol. **4**, 167–180
28 Spear, P. G., Eisenberg, R. J. and Cohen, G. H. (2000) Three classes of cell surface receptors for alphaherpesvirus entry. Virology **275**, 1–8
29 Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D. and Spear, P. G. (1999) A novel role for 3-*O*-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell **99**, 13–22
30 Berninsone, P. and Hirschberg, C. B. (1998) Heparan sulfate/heparin *N*-deacetylase/*N*-sulfotransferase. The *N*-sulfotransferase activity domain is at the carboxyl half of the holoenzyme. J. Biol. Chem. **273**, 25556–25559
31 Kakuta, Y., Pedersen, L. G., Pedersen, L. C. and Negishi, M. (1998) Conserved structural motifs in the sulfotransferase family. Trends Biochem. Sci. **23**, 129–130
32 Kakuta, Y., Sueyoshi, T., Negishi, M. and Pedersen, L. C. (1999) Crystal structure of the sulfotransferase domain of human heparan sulfate *N*-deacetylase/*N*-sulfotransferase 1. J. Biol. Chem. **274**, 10673–10676
33 Hernaiz, M., Liu, J., Rosenberg, R. D. and Linhardt, R. J. (2000) Enzymatic modification of heparan sulfate on a biochip promotes its interaction with antithrombin III. Biochem. Biophys. Res. Commun. **276**, 292–297
34 Zhang, L., Schwartz, J. J., Miller, J., Liu, J., Fritze, L. M., Shworak, N. W. and Rosenberg, R. D. (1998) The retinoic acid and cAMP-dependent up-regulation of 3-*O*-sulfotransferase-1 leads to a dramatic augmentation of anticoagulantly active heparan sulfate biosynthesis in F9 embryonal carcinoma cells. J. Biol. Chem. **273**, 27998–28003
35 Shworak, N. W., Shirakawa, M., Mulligan, R. C. and Rosenberg, R. D. (1994) Characterization of ryudocan glycosaminoglycan acceptor sites. J. Biol. Chem. **269**, 21204–21214
36 Montgomery, R. I., Warner, M. S., Lum, B. J. and Spear, P. G. (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87**, 427–436
37 Nagata, K. and Yamazoe, Y. (2000) Pharmacogenetics of sulfotransferase. Annu. Rev. Pharmacol. Toxicol. **40**, 159–176
38 Marsolais, F., Laviolette, M., Kakuta, Y., Negishi, M., Pedersen, L. C., Auger, M. and Varin, L. (1999) 3′-Phosphoadenosine 5′-phosphosulfate binding site of flavonol 3-sulfotransferase studied by affinity chromatography and ^{31}P NMR. Biochemistry **38**, 4066–4071
39 Sueyoshi, T., Kakuta, Y., Pedersen, L. C., Wall, F. E., Pedersen, L. G. and Negishi, M. (1998) A role of Lys614 in the sulfotransferase activity of human heparan sulfate N-deacetylase/N-sulfotransferase. FEBS Lett. **433**, 211–214
40 Petrotchenko, E. V., Doerflein, M. E., Kakuta, Y., Pedersen, L. C. and Negishi, M. (1999) Substrate gating confers steroid specificity to estrogen sulfotransferase. J. Biol. Chem. **274**, 30019–30022
41 Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M. and Pedersen, L. C. (1997) Crystal structure of estrogen sulphotransferase. Nat. Struct. Biol. **4**, 904–908
42 Marsolais, F. and Varin, L. (1995) Identification of amino acid residues critical for catalysis and cosubstrate binding in the flavonol 3-sulfotransferase. J. Biol. Chem. **270**, 30458–30463
43 Wei, Z. and Swiedler, S. J. (1999) Functional analysis of conserved cysteines in heparan sulfate *N*-deacetylase-*N*-sulfotransferases. J. Biol. Chem. **274**, 1966–1970
44 Hong, W. and Tang, B. L. (1993) Protein trafficking along the exocytotic pathway. BioEssays **15**, 231–238
45 Humphries, D. E., Sullivan, B. R., Aleixo, M. D. and Snow, J. L. (1997) Localization of human heparan glucosaminyl *N*-deacetylase/*N*-sulphotransferase to the *trans*-Golgi network. Biochem. J. **325**, 351–357

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